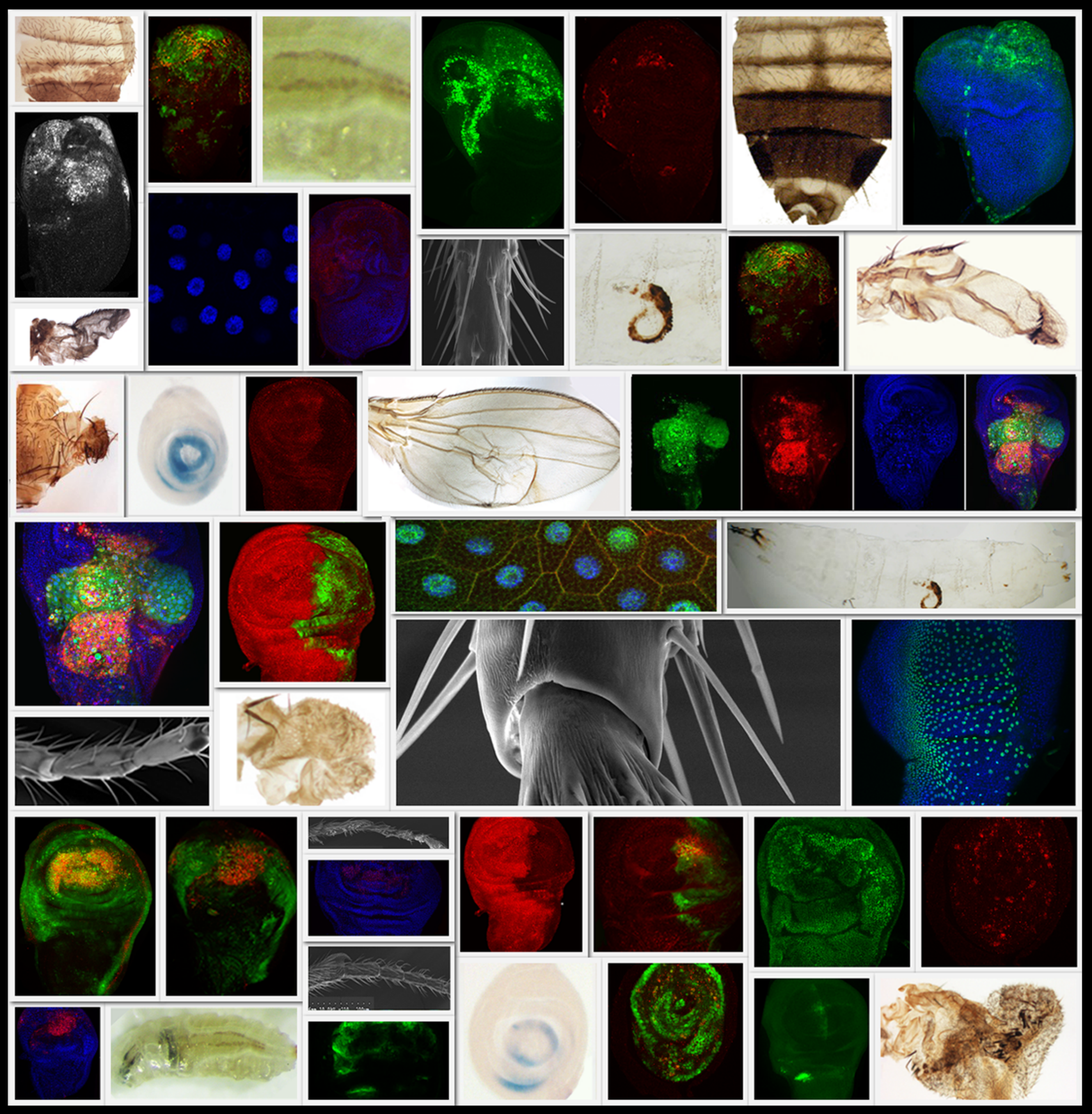
dRYBP transcription-dependent and transcription-independent functions in *Drosophila* development



Sol Fereres Rapoport

Doctoral Thesis

Madrid, 2014

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE BIOLOGÍA MOLECULAR

dRYBP transcription-dependent and transcription-independent functions in *Drosophila* development

Thesis Director: Ana de Busturia Jimeno

Centro de Biología Molecular Severo Ochoa (UAM-CSIC)

Thesis submitted by Sol Fereres Rapoport to fulfill the requirements of the Doctorate Degree in Molecular Biology from the Universidad Autónoma de Madrid

This research work was carried out under the supervision of Dr. Ana de Busturia Jimeno, in the Centro de Biología Molecular Severo Ochoa (CSIC-UAM).
This work was supported by a fellowship from the Universidad Autónoma de Madrid for Graduate Studies (2010/11), by a fellowship from the Fundación Severo Ochoa for Pre-doctoral research (2011), by grants from the Dirección General de Investigación (BFU-2008-01154) to A.B. (2012), the Consolider Ingenio 2010 Program of the Ministerio de Ciencia e Innovación (CSD2007-0008) to A.B. (2013), and by a short-term Fellowship from EMBO (2013).

To Sonia, my number one fan

First of all, I would like to thank my supervisor, Ana for giving me the opportunity of doing my thesis in her laboratory. Thank you for trusting that shy girl that entered your office about five years ago, believing in her and for showing her that a thesis is not only about asking questions and trying to find the answers. Thank you for your patience and for teaching me about science but, most importantly, about life. For treating me like family. Thank you, Ana.

To my parents, Elías and Hava, for their unconditional support and for always encouraging me to do whatever I wanted. For putting up with me during all that time when I was insecure and didn't know what to do and for letting me make my own decisions (even if they weren't the right ones). Thank you for believing in me, this wouldn't have been possible without you. To Sonia, my big sister, my number one fan, who has always taken good care of me, even overseas and miles away. Thank you for making me work that summer at UC Berkeley when I was visiting you. I owe you my first research experience at Prof. Jeremey Thorner's lab and learning that this is what I wanted to do. To Eirun, for looking out for me while Sonia wasn't here and to little Henry, who still doesn't know it but has made me smile every time I see him since he was born and has made all the writing process so much more fun! Who knows? Maybe he will continue with our family research tradition (sorry Eirun, it runs in the genes!). Special thanks to my grandpa Henry, I can still remember how he would talk during dinner time about how he was dealing with his current research challenges and how exciting science could be, and to my grandma Sonya, with whom I have shared very special moments discussing about art and science and for encouraging me to learn how to use Photoshop! To all of my family that has supported me in one way or the other, thank you.

To all the members of the 411 laboratory in Madrid, without you it wouldn't have been the same. To Richi, the lab master, thank you for your infinite patience, for helping me with every question I had during this time, even if I was repetitive and annoying, and for even helping me by email when I was miles away. To Rocío, my mentor, thank you for teaching me everything you know, for putting up with all of my questions, for sometimes being a therapist and dealing with all of my problems and for making my life more fun. It was good to start work on Mondays just to see you and talk. To Carol, for dealing with my moments of crisis and for stopping whatever you were doing just to help me, for all your advice and of course, thank you for your contagious laugh, you are responsible for some of the best moments in the lab. Thank you so much all of you and other former members for making the lab such a fun and friendly environment and for creating a small family, as we say: "in four-eleven you're in heaven!"

To the 425 lab, Alicia, Javi and Marta for all of your support and great moments together and in general, to all the fly community for helping out, for fruitful discussions and for just making it easier and better.

ACKNOWLEDGEMENTS

To Prof. C. Peter Verrijzer for giving me the opportunity of going to his laboratory in Rotterdam for three months and learning more than I could ever imagine. Thank you Adone and Diana for all your help and for making me enjoy my stay in Rotterdam. Diana, I would have never thought making gels could be so much fun!

To Prof. Hermann Steller for giving me the chance to go to his laboratory in New York and helping me develop as a scientist. To the Steller laboratory for all their help, especially to Joe, Xiaoling, Yaron, Park and Cristi. To Sigi for dealing with me and my experiments when she didn't have to and for pushing me to continue when things weren't working out. Special thanks to the Spanish crew: Ainhoa, for taking care of me, Fernando, Dolors and Celia, you guys made lunch the best part of the day and certainly made me enjoy so much my months in New York, it wouldn't have been the same without you.

To my classmates Vero, Celia, Alberto, Julia and Anais, you have all been great friends these past years. I would like to give special thanks to Vero, for putting up with me during our first lab course even when I was a complete disaster, if it weren't for her who knows if we would all be friends. Thank you everybody for being there when I needed you, for great moments and not only on campus, for happy hours at Friday's, for all of your visits to the lab and different places and for listening.

To Javi and Reyes, for our "piggy lunches" in the CBM, you have certainly have made a difference. Thank you for making me laugh so much and for making more enjoyable my time at the CBM.

To my friends Cae, Marga, Alex, Javi and Maria del mar, who always tried, although I am not sure they did, to understand what I was doing. Thank you guys for helping me disconnect and for sharing wonderful moments along these years.

And last but not least, I would like to thank Javi, for standing by me all of these past years, especially the last few months. Thank you for learning about science and for coming to the lab and helping me with my fly stocks, for listening, even when you didn't know what I was talking about and for calming me down when things weren't working out. Thank you for making me have fun, for all our trips around the world and for making me happier.

"Anyone can do science when the experiment works out,

it takes a scientist when it doesn't"

Prof. Henry Rapoport, University of California, Berkeley

INDEX

INDEX

INDEX

Abbreviations

Amp	Ampicilin
Ant-C	Antennapedia Complex
AP	Alkaline phosphatase
Ark	Apaf-1 related killer
ASH1	Absent, small o homeotic discs 1
ASH2	Absent, small o homeotic discs 2
β-gal	Beta-galactosidase
BRM	Brama
BSA	Benzenesulfonic acid
BX-C	Bithorax complex
C3	activated Caspase-3
COMPASS	Complex Proteins Associated with Set1
Dcp-1	*Drosophila* caspase-1
DD	Death domain
DED	Death efector domain
DIAP1	*Drosophila* inhibitor of apoptosis 1
dKDM2	*Drosophila* Lysine (K)-specific demethylase 2
DMSO	Dimethyl sulfoxide
dNE	*Drosophila* nuclear extracts
DOC	2,5-Dimethoxy-4-chloroamphetamine
dRAF	dRING associated factors
dRB	*Drosophila* RYBP-BRE1 complex
Drice	*Drosophila* interleukin 1 beta-converting enzyme
Dronc	*Drosophila* Nedd2-like caspase
dRRK	*Drosophila* RING-RYBP-KDM2 complex
dRYBP	*Drosophila* Ring1B Yin and yang1 binding protein
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESC	Extra sex combs
ESCL	Extra sex combs-like
EZ	Enhancer of zeste
GST	Glutathione S-transferase
h	hour
HEMG	Protoporphyrinogen IX dehydrogenase (menaquinone)
HRP	Horseradish peroxidase
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth (Luria-Bertani)
Mass-spec	Mass Spectometry
Me	methylation
Me2	dimethylation
Me3	trimethylation
min	minutes
mRNA	messenger RNA
MW	Molecular weight
NP-40	Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol)

NZF	Nucleoporin Zinc finger
o/n	overnight
Pall	Pallbearer
PBS	Phosphate Balanced Saline
PBT	Phosphate Balanced Saline and Triton
PC	Polycomb
PcG	Polycomb-group proteins
PCL	Polycomb-like
PCR	Polymerase chain reaction
PH	Polyhomeotic
PHO-RC	Pleihomeotic repressive complex
PHO	Pleihomeotic
PHOL	Pho-like
PMSF	Phenylmethylsulfonyl fluoride
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PRE	Polycomb response element
PSC	Posterior sex comb
RT	Room temperature
RTqPCR	Retro-trancriptase quantitative polymerase chain reaction
SCE/dRING	Sex comb extra/ Drosophila Really interesting new gene
SCF	Skp1-Cullin-F-box complex
SCM	Sex comb on midleg
Scr	Sex comb reduced
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SET	Su(var)3-9 and Enhancer of zeste protein
SKPA	S-phase kinase-related protein A
Su(z)12	Suppressor of zeste 12
SWF/SNF	Switch/Sucrose Non Fermentable
TRE	Trithorax response element
Trl	Trithorax-like
Trr	Trithorax-related
Trx	Trithorax
trxG	Trithorax Group
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UBD	Ubiquitin binding domain
UIM	ubiquitin-interacting motif
WB	Western Blot

Summary

In this Thesis we have analyzed, using *Drosophila* as a model system, the function of *dRYBP* (drsosophila Ring and YY1 binding protein) in the control of gene expression. The genetic and biochemical analysis of dRYBP reveals its role in the epigenetic control of gene expression and in the regulation of cell death. dRYBP controls these processes by two different mechanisms: one, a transcription-dependent mechanism whereby dRYBP, together with the Polycomb and trithorax proteins, modulates levels of post-translationally modified histones. The second one, a transcription-independent mechanism whereby dRYBP regulates the apoptotic pathway through its interaction with the SCF E3 ubiquitin ligase complex, involved in proteasomal degradation of target proteins. The conclusions of this Thesis are: 1) The dRYBP protein coexists, in *Drosophila* S2 cells, in two different forms: the dRYBP and the monoubiquitylated dRYBP (dRYBPub) proteins. Moreover, the dRYBP protein binds to ubiquitylated proteins through its N-terminal, where the NZF domain is located. 2) The dRYBP protein interacts with the H2A, H2B, H2Aub and H2Bub histones in *Drosophila* S2 cells. Additionally, *dRYBP* genetically interacts with *Sce/dRing*, *dkdm2* and *dBre1* and biochemically, in *Drosophila* wild type embryonic nuclear protein extracts (dNE), with SCE/dRING, dKDM2 and dBRE1. 3) The dRYBP protein does not biochemically interact with the PSC, PC, PH and EZ proteins and dBRE1 does not interact with the SCE/dRING, dKDM2 and EZ proteins in dNE. 4) Inactivation of *dRYBP* decreases levels of monoubiquitylated H2A (H2Aub) and monomethylated H3K4 (H3K4me). 5) dRYBP counteracts dKDM2-mediated H3K36me2 demethylation and dBRE1-mediated H2B monoubiqutylation. These dRYBP-mediated activities may therefore attenuate *dkdm2*-mediated repression and *dBRE1*-mediated activation. 6) The *dRYBP* gene interacts genetically with *skpA*, *dCul1* and *slmb*, all members of the SCF E3 ubiquitin ligase complex. Moreover, the dRYBP protein biochemically interacts with SKPA and dCUL1 proteins. 7) Inactivation of *dRYBP, skpA, dCul1* and *slmb* induces apoptosis in the wing imaginal disc and the inactivation of *skpA*- and *dRYBP*-induced apoptosis is dependent on the expression levels of the pro-apoptotic gene *rpr* and the anti-apoptotic protein DIAP1. Thus the dRYBP-SCF complex functions by inhibiting the intrinsic apoptotic pathway. 8) Inactivation of *skpA* in wing imaginal discs and in *Drosophila* S2 cells, induces the transcriptional activation of *rpr* and *diap1*, increases Rpr protein levels and decreases DIAP1 protein levels. 9) High levels of SKPA rescues the apoptotic wing phenotype induced by overexpression of Rpr. Moreover, in human HEK293 cells, SKPA protein biochemically interacts with Rpr and DIAP1 proteins and SKPA overexpression decreases Rpr protein levels, suggesting that the dRYBP-SCF complex post-translationally regulates Rpr protein levels. 10) High levels of SKPA inhibit both the apoptosis that occurs during normal leg development and X-ray-induced apoptosis.

Introduction

1. *Drosophila melanogaster* AS A MODEL SYSTEM

Developmental biology studies the mechanisms underlying the growth and development of a single cell, the fertilized oocyte, to an adult organism. The fruit fly *Drosophila melanogaster* is one of the most relevant model systems used to analyze the mechanisms controlling the genetic regulation of cell proliferation, differentiation and morphogenesis. This is due to numerous advantages among which we find it is small size and easy to grow, it has a short life cycle, it has a high fecundity and only four chromosomes compose the whole genome. Moreover, the *Drosophila* genome is phylogenetically conserved. The identity at the nucleotide level between *Drosophila* and humans is approximately 40%, however the conservation in functional domains can approximately be 80% to 90% or higher (Pandey and Nichols, 2011). Interestingly 75% of human disease genes have a homolog in *Drosophila* (Reiter et al., 2001) and importantly the signaling pathways controlling development are also evolutionary conserved (Vidal and Cagan, 2006). Additionally, the use of *Drosophila* as a model system for studying developmental biology has allowed the generation of genetic tools, such as a library of transgenic flies that allows gene overexpression and inactivation, that are not available for other model organisms confirming once more, the advantages of *Drosophila* as a model system.

The life cycle of *Drosophila melanogaster* takes place in ten days approximately at 25ºC and goes through different developmental stages (Figure 1A).

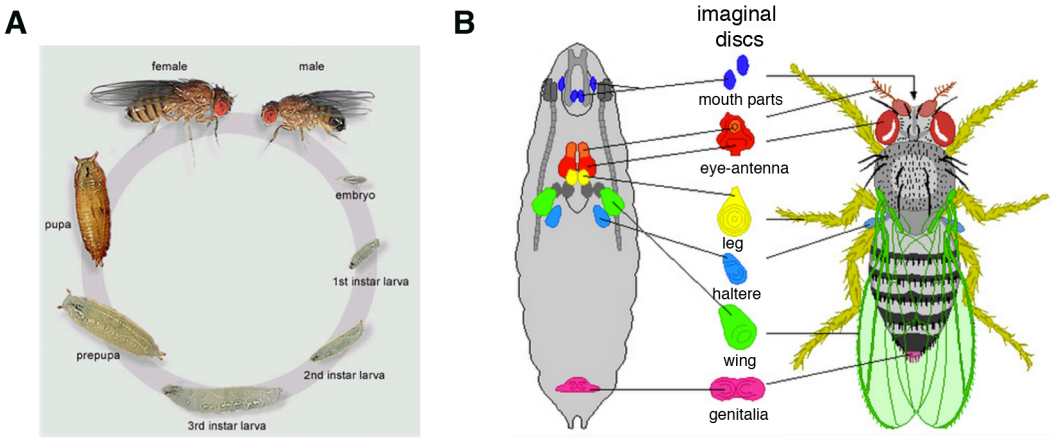


Figure 1. Development of *Drosophila melanogaster*. (**A**) Life cycle of *Drosophila melanogaster* (**B**) Larva showing salivary glands and imaginal discs and the structure they will give rise to after metamorphosis in the adult fly. (Modified from Flymove).

The first stage is the embryonic phase, which lasts approximately twenty-four hours. Then, after hatching the resulting larvae grow for about four days while molting twice (to 2nd and to 3rd instar larvae). During the larval phase proliferation of the imaginal discs, the

primordia of the structures of the adult body, takes place (Figure 1B). The last period of the life cycle that also lasts approximately four days, corresponds to the pupal stage, where metamorphosis takes place and the abdominal histoblasts proliferate to generate the adult abdomen. During metamorphosis many larval tissues are reabsorbed and the imaginal discs undergo morphogenetic changes to form adult structures, for example the wing imaginal discs will generate the adult wing, the hinge and the thorax (Figure 1B). Afterwards the adult fly emerges (Ashburner, 1989).

1.1. Genetic control of *Drosophila* morphogenesis

The body of *Drosophila*, as many other insects and organisms is segmented. Segmentation occurs very early in development and is controlled by a genetic cascade that includes many transcription factors that function sequentially during embryonic development (Figure 2). First, the anterior-posterior (A/P) axis is established by the maternal genes, such as *bicoid* and *nanos*, which provide the positional information (Lawrence, 1992). The maternal genes, in turn, control the expression of the gap genes, such as *Kruppel*, that are expressed in broad domains along the A/P axis and, in turn, control the expression of the pair rule genes (Lawrence, 1992) (Figure 2).

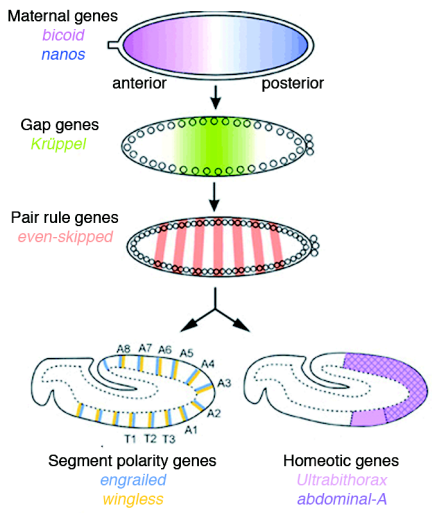


Figure 2. Segmentation of the *Drosophila melanogaster* embryo. The maternal proteins activate the differential expression pattern of the gap genes, which activate the expression of the pair rule genes, which activate the expression of the segment polarity genes and coordinately activate the expression of the homeotic genes. See text for more detailed explanations.

The pair rule genes are differentially expressed in seven alternative stripes that will allocate cells to the prospective 14 parasegments (PS). The parasegments are the metameric functional units and are formed by the posterior compartment of one segment and the anterior compartment of the following segment (Martinez-Arias and Lawrence, 1985). For example the PS6 is composed of the posterior compartment of the T3 segment and the anterior compartment of the A1 segment (Figure 3). Once the number of parasegments has been established, the segment polarity genes, such as *engrailed* and *wingless*, specify the anterior and posterior compartments within each parasegment (Lawrence, 1992) (Figure 2). The pair rule genes as well as the segment polarity genes regulate the expression of the homeotic (*Hox*) genes, which are responsible for the cellular identity of each segment throughout

development (Lawrence, 1992) (Figure 3). Moreover, the homeotic genes specify the segmental identity of the cephalic, thoracic and abdominal segments and will ultimately, be responsible for the identity of the adult structures and body pattern of the fly (Lewis, 1978).

The *Hox* genes codify for transcription factors, evolutionary conserved, that regulate the expression of other genes organizing the development of the mesoderm, endoderm and ectoderm, the nervous system and the epidermis of many animals, from fruit flies to vertebrates (Akam et al., 1994; Krumlauf, 1994). The *Hox* genes are clustered in the Antennapedia (Ant-C) and Bithorax complexes (BX-C) (Figure 3). For instance, the *Scr* (*Sex combs reduced*) gene belongs to the Ant-C (Duncan, 1987; Kaufman et al., 1990) and the BX-C is composed of the *Ubx* (*Ultrabithorax*), *abd-A* (*abdominal A*) and *Abd-B* (*Abdominal B*) genes (first described in (Sanchez-Herrero et al., 1985a)) (Figure 3). As pointed out above, the expression domains of the *Hox* genes are established along the A/P axis very early during embryonic development. These domains define the cells where the *Hox* genes are expressed, "expression domains" and importantly, they also define the cells where the *Hox* genes are repressed, "repression domains" to allow normal development (Lawrence, 1992). For instance, the expression of the *Ubx* gene in the 3rd dorsal thoracic segment promotes the development of a haltere, while the 2nd dorsal thoracic segment, where *Ubx* is repressed, develops into a wing (Sanchez-Herrero et al., 1985b).

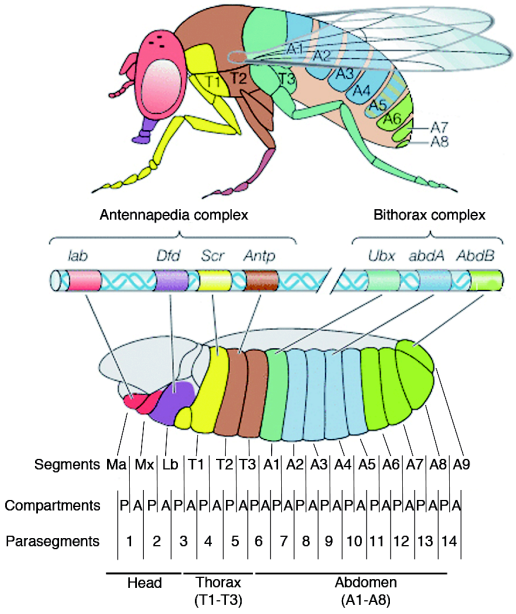


Figure 3. Homeotic genes. Simplified representation of the proximal-distal localization of the *Hox* genes in the chromosome and their expression domains throughout the anterior-posterior axis of the *Drosophila* embryo and adult body. In addition the segments, compartments and parasegements of the embryo are indicated. See text for more detailed explanations.

Both the "expression" and "repression" domains have to be maintained throughout development because alterations, at any time of development in the expression pattern of the *Hox* genes can lead to lethality in most cases, or severe phenotypic transformations. Importantly these expression domains of the *Hox* genes are established by the segmentation genes (the gap and the pair rule genes), which are expressed only during the first four hours of development (Jackle et al., 1992), while the *Hox* genes are expressed and required

throughout development. Thus, the maintenance of the expression domains of the homeotic genes is achieved by another set of genes: the *Polycomb* (*PcG*) and *trithorax (trxG)* groups of genes, in charge of maintaining throughout development the repressed and activated transcriptional states of the *Hox* genes respectively (Kennison and Tamkun, 1988; Lewis, 1978; Simon et al., 1992).

The PcG and trxG are nuclear proteins that are ubiquitously expressed throughout development. Initially discovered in *Drosophila* as regulators of the *Hox* genes, it is now known that they are evolutionary conserved and that they regulate the expression of many other genes involved in diverse biological processes such as proliferation, stem cell pluripotency and tumorigenesis (Ferres-Marco et al., 2006; Martinez et al., 2006; Oktaba et al., 2008; Probst et al., 2009). There are at least 40 genes that have been described as *PcG* and *trxG* genes. These genes codify for proteins with different domains that function in multiprotein complexes composed of either PcG or trxG proteins. These complexes modulate chromatin condensation by post-translationally modifying histones leading to transcriptional repression in the case of PcG proteins or transcriptional activation in the case of trxG proteins (Schuettengruber and Cavalli, 2010; Schwartz and Pirrotta, 2007). Additionally, within the described PcG and trxG, there is a set of genes that codify for proteins that interact with both PcG and trxG proteins and are known as ETPs (Enhancers of Trithorax and Polycomb) (Bejarano and Busturia, 2004; Busturia et al., 2001; Gildea et al., 2000). Among the ETPs we find the dRYBP (drosophila Ring1B Yin yang1 binding protein) protein (Bejarano et al., 2005; Gonzalez et al., 2008), object of study of this thesis work and which will be described in more detail in section 3.2. of the introduction.

2. REGULATION OF GENE EXPRESSION

All organisms seek for homeostasis to reach a correct development and maintain a healthy life. This is accomplished through an active response to external and internal signals that ultimately allow organisms to survive against harmful attacks. A miss-regulation of gene expression during both development and the adult life can lead to diseases such as cancer or neurodegenerative disorders (Bray et al., 2005; Ferres-Marco et al., 2006). Hence an accurate regulation of gene expression is crucial during development to achieve correct morphogenesis.

Regulation of gene expression occurs at different stages underlying different molecular mechanisms at the level of chromatin, DNA, RNA, translation and also at the post-translational level. For example, chromatin condensation, which can be altered as a result of histone post-translational modifications, results in a transcriptional activation or repression of

gene expression (Hubner et al., 2013). Also, DNA transcription can be regulated by different mechanisms. One of them is mediated by transcription factors that control the temporal and spatial regulation of gene expression by physically binding to cis-regulatory enhancers or silencers present at the DNA targets, altering the specificity of the RNA polymerase for a given promoter (Gaarenstroom and Hill, 2014). Moreover, post-transcriptional regulation includes mRNA modifications, including alternative splicing, addition of the poly(A) tail (Xu et al., 1994), or regulation by micro-RNAs (Ambros, 2004). Translation can also be regulated by the secondary structure of the mRNA or protein binding (Cheadle et al., 2005). Furthermore, proteins can be post-translationally modified by the attachment of one or several biochemical groups, such as ubiquitin, modifying the nature and/or structure of the target protein (Mann and Jensen, 2003). Consequently post-translational modifications change protein activity promoting their degradation, activation, inactivation or changing its cellular localization (Inuzuka et al., 2011; Kulathu and Komander, 2012; Mann and Jensen, 2003; Osley, 2004). Several post-translational modifications regulate protein activity like phosphorylation, methylation, sumoylation and protein ubiquitylation, object of study of this thesis work.

2.1. Protein ubiquitylation

Protein ubiquitylation is a post-translational modification that involves a series of catalytic reactions resulting in the covalent attachment of one (mono-) or several (poly-) molecules of ubiquitin to a lysine residue in the target protein (Ciechanover et al., 1980; Hershko et al., 1979). Apart from the lysine residues in the target protein, the ubiquitin molecule can be a target itself for ubiquitylation. It has seven lysine residues: K6, K11, K27, K29, K33, K48 and K63, opening the possibility of forming different type of linkages depending on the lysine that is used for ubiquitin chain elongation that can affect protein function in many different ways, such as endocytosis, proteolysis and trafficking (Kulathu and Komander, 2012) (Figure 4). Additionally proteins can non-covalently bind to a ubiquitin molecule or poly-ubiquitin chain that has been previously attached to a target protein, in order to transmit the information conferred in protein ubiquitylation. This occurs through ubiquitin binding domains (UBD), which are protein domains structurally diverse that recognize ubiquitylated targets (Hicke et al., 2005) (Table 1).

Table 1. Ubiquitin binding domains (UBD)

Domain	Length (aa)	Ubiquitin-binding affinity (Kd in μM)
CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation)	42-43	2-160μM (binds monoubiquitin)
GAT (Gga and TOM1)	135	180μM (monoubiquitin)
GLUE (GRAM-like ubiquitin-binding in Eap45)	~135	460μM (binds monoubiquitin)
NZF (Nucleoporin Zinc Finger)	35	100-400μM (binds monoubiquitin)
PAZ (polyubiquitin-associated zinc finger)	58	Not known
UBA (Ubiquitin-associated domain)	45-55	10-500μM (binds monoubiquitin) 0.03-9mM (binds polyubiquitin)
UEV (ubiquitin-conjugating enzyme variant)	~145	100-500μM (binds monoubiquitin)
UIM (ubiquitin-interacting motif)	~ 20	100-400μM (binds mono and polyubiquitin)
VHS (Vps27, HRS, STAM)	150	Not known

The ubiquitylation pathway involves three different steps carried out by three different enzymes (Figure 4). The first one is the E1 ubiquitin-activating enzyme that forms, in an ATP-dependent manner, a thioester linkage between the ubiquitin and itself. The second step, carried out by the E2 ubiquitin-conjugating enzyme, catalyzes the transfer of ubiquitin from the E1 to the active-site cysteine of the E2. The third and final step is executed by the E3 ubiquitin ligase enzyme and results in the formation of an isopeptide bond between the C-terminal of the ubiquitin and the lysine residue of the target protein (Ciechanover et al., 1982; Hershko et al., 1983). The ubiquitylation pathway is evolutionary conserved from fly to humans (Bocca et al., 2001; Zhang and Sidhu, 2014). The variety of proteins involved makes the process of ubiquitylation very diverse and complex but also very specific in order to ensure the attachment of the right number of ubiquitin molecules to the intended substrate in the correct position. There exists one gene in *Drosophila* (*uba1)*, and two functional homolog genes in humans that codify for the E1. There are few more genes that codify for the E2 in *Drosophila* and humans and many more that encode for the E3.

One of the most important superfamilies of E3 ubiquitin ligases is the SCF (SKP1-CUL1-F-box) (Cardozo and Pagano, 2004). This complex consists of a scaffold protein, Cullin1 (CUL1), whose C-terminal binds to a RING domain containing protein, and whose N-terminal binds to the adaptor protein SKP1 (S-Phase associated-Protein 1) that interacts with a F-box-containing protein (Jackson and Eldridge, 2002). The F-box-containing protein is the subunit responsible for substrate specificity by recruiting the target protein (Bocca et al., 2001; Skowyra et al., 1997). The SCF complex, originally identified in *S. cerevisiae,* is evolutionarily conserved (Feldman et al., 1997) and has been shown to regulate multiple

physiological pathways including those involved in metabolism (Sundqvist et al., 2005) apoptosis (Inuzuka et al., 2011; Tan et al., 2006) and genome stability (Silverman et al., 2012).

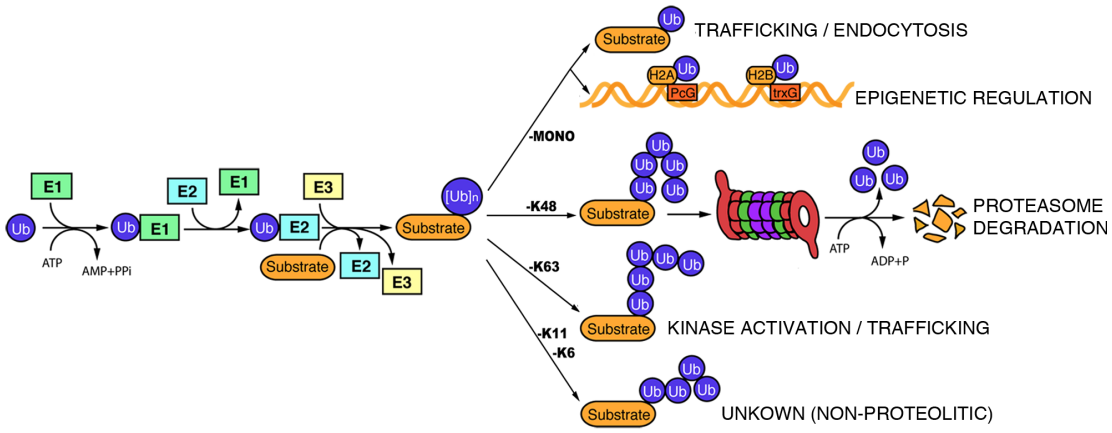


Figure 4. The ubiquitylation pathway. The coordinate action of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase enzyme results in the covalent attachment of one (mono) or several (poly) ubiquitin molecules (Ub) to the target protein (substrate). Protein ubiquitylation results in different physiological outcomes depending on the number of ubiquitin molecules attached to the substrate and the type of linkage. See text for more detailed explanations.

Different types of ubiquitin linkages have different physiological outcomes (Figure 4). For instance, monoubiquitylation is involved in epigenetic regulation of gene expression (Tanny et al., 2007; Wang et al., 2004) while -K48 type of linkage has been shown to promote proteasomal degradation of the targeted protein (Ciechanover et al., 1980), a very relevant process to maintain proteins levels, in particular in the process of apoptosis (Bergmann, 2010; Vucic et al., 2011). The mechanisms regulating the epigenetic control of gene expression and the mechanisms involved in the regulation of the apoptotic signaling pathway will now be described.

3. EPIGENETIC REGULATION OF GENE EXPRESSION MEDIATED BY PcG AND trxG

The mechanisms of action of PcG and trxG proteins involve three main steps (Figure 5). 1) Binding of either PcG and trxG proteins containing specific DNA binding domains, such as PHO (Pleiohomeotic) and Trl (Trithorax-like) to specific DNA sequences called PRE/TREs (Polycomb and Trithorax Response Elements) found at the target genes. 2) Recruitment of PcG and trxG proteins to DNA to form specific multiprotein complexes. 3) Histone post-translational modifications, such as methylation, acetylation and ubiquitylation,

mediated by the different biochemical activities of each multimeric complex (Figures 5 and 6), leading to different states of chromatin condensation and hence, different levels of gene transcription (Klymenko et al., 2006; Muller and Kassis, 2006; Schuettengruber and Cavalli, 2010).

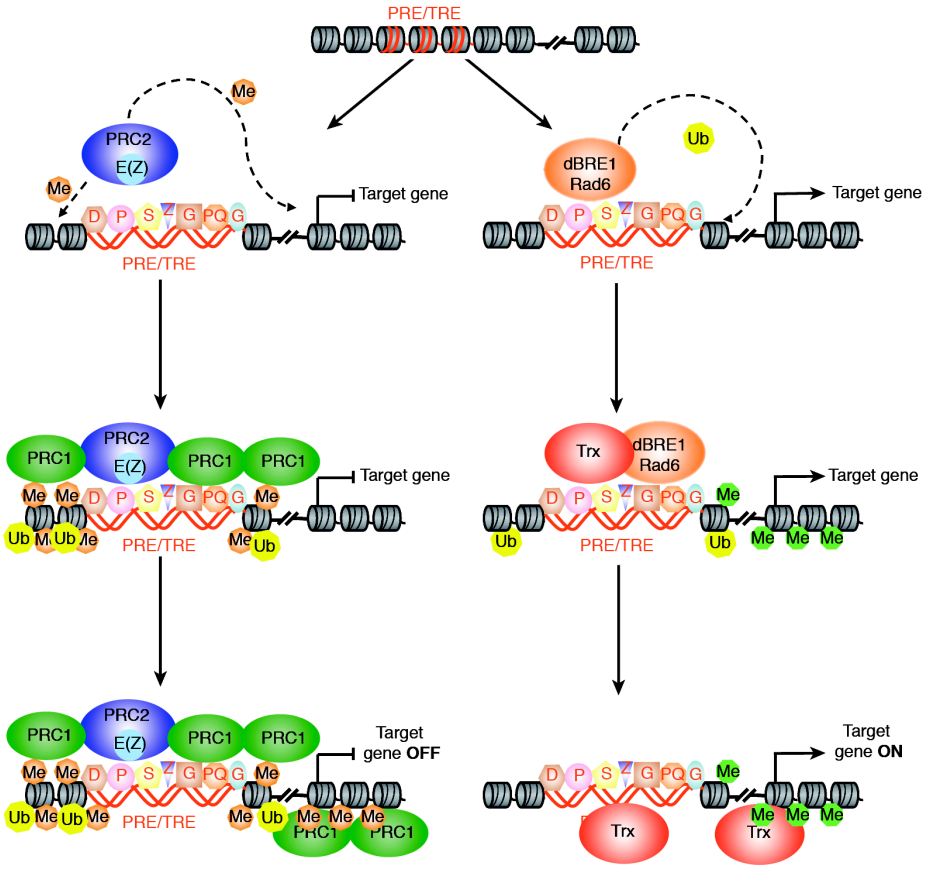


Figure 5. Mechanism of action of the PcG and trxG proteins. The PcG and trxG proteins are recruited to the PREs and TREs (Polycomb/Trithorax Response Elements) in their target genes by their interactions with DNA-binding proteins such as PHO and GAF. For gene repression the PRC2 complex, trimethylates (Me) H3K27 through E(z). This mark is recognized by the chromodomain of Pc of the PRC1 complex, which is then recruited to ubiquitylate (Ub) H2A. The coordinate action of the PRC2 and PRC1 complexes leads to transcriptional repression of the taget gene. For gene activation the dBRE1 protein dBRE1 together with the E2 enzyme Rad6 ubiquitylate (Ub) H2B. This mark recruits other trxG protein complexes (Trx) which di- and tri-methylate H3K4, leading to transcriptional activation of target genes.

3.1. PcG and trxG complexes and their biochemical activity

PcG and trxG proteins (Tables 2 and 3) assemble at PREs into different multimeric complexes, each of one with a specific biochemical activity that post-translationally modifies histones (Figure 6), which ultimately translates into transcriptional gene repression or activation respectively (Muller and Verrijzer, 2009). Some of the best-characterized PcG complexes (Table 2), of interest for this thesis work, are the PRC1 (Polycomb Repressive

Complex 1) (Shao et al., 1999), the PRC2 (Polycomb Repressive Complex 2) (Czermin et al., 2002; Muller et al., 2002) and the dRAF (dRing Associated Factors) (Lagarou et al., 2008).

Table 2. Composition of PcG protein complexes

Complex	*Drosophila* Protein	Human homolog	Domain	Biochemical activity
PRC1	Polycomb (PC)	CBX2, 4, 6, 7 and 8	Chromodomain	Binds H3K27me3
PRC1	Polyhomeotic (PH)	PHC1,2 y 3	Zn finger, SAM	
PRC1/dRAF	Posterior sex comb (PSC)	PCGF1(NSPc1), PCGF2(MEL18), PCGF4(BMI1)	Zn finger	H2AK119 ubiquityl ligase
PRC1/dRAF	dRING/Sex combs extra (SCE)	RING1A, RING1B, RNF2	RING Zn finger	H2AK119 ubiquityl ligase
PRC1-associated factors	Sex comb on midleg (SCM)	SCMH1, SCML2	Zn finger, SAM, MBT	Protein recruiter PcG
PRC2	Enhancer of zeste E(z)	EZH1y 2	SET	H3K9 y H3K27 methyl transferase
PRC2	Extra sex combs (ESC)	EED	WD40	Cofactor for E(z)
PRC2	Suppressor of zeste 12 [SU(Z)12]	SUZ12	Zn Finger	Cofactor for E(z)
PRC2-associated factors	Polycomb-like (PCL)	PCL1(PHF1), PCL2(MTF2), PCL3(PHF19)	PHD	
PRC2-associated factors	Extra sex comb-like (ESCL)	EED	Repeticiones WD40	Cofactor for E(z)
PRC2-associated factors	SIR2	SIRT1	Zn finger	Histone deacetyl transferase dependant on NAD^+
dRAF	dKDM2	KDM2B, FBXL10	CXXC Zn finger, JmjC, Fbox	H2AK119 ubiquityl ligase, H3K36me2 demethyl transferase
PhoRC	Pleihomeotic (PHO)	YY1 y 2	Zn finger	Binds DNA
PhoRC	Pho-like (PHOL)	YY1 y 2	Zn finger	Binds DNA
PhoRC	Scm-related gene containing Four Mbt domains (dSFMBT)	L3MBTL2, MBTD1	MBT, SAM	Binds H3K9me1, me2 and H4K20me1 me2
PR-DUB	Calypso/dBAP1	BAP1	UCH	H2AK119 de-ubiquityl transferase
PR-DUB	Additional sex comb (ASX)	ASXL1		Cofactor for dBAP1

The core components of the PRC1 complex are the PC (Polycomb), PH (Polyhomeotic), PSC (Posterior Sex Comb) and SCE/dRING (Sex Comb Extra) proteins (Shao et al., 1999). In addition to these core members, other proteins can associate to PRC1, such as Zeste or Scm (Sex comb on midleg), which enhance the repressive activity of the PRC1 (Mulholland et al., 2003; Muller and Kassis, 2006)}. The PRC1 is responsible for monoubiquitylating histone H2A in K119 (K118 in *Drosophila*), activity that is mediated by the E3 ubiquitin ligase RING domain of the protein SCE/dRING (Buchwald et al., 2006; de Napoles et al., 2004; Gutierrez et al., 2012; Lagarou et al., 2008) (Figure 6). Additionally, PC has a

chromodomain that specifically binds to the trimethylated H3K27 (H3K27me3), a histone modification established by PRC2 (Fischle et al., 2003).

The PRC2 is composed by the EZ (Enhancer of Zeste), ESC (Extra Sex Comb), Su(Z)12 (Suppresor of Zeste 12) and Nurf55 (Nucleosome remodeling factor 55) proteins. This complex is responsible for the trimethylation of the H3K27, the modification that recruits PRC1 through its interaction with the chromodomain contained in the PC protein (Czermin et al., 2002; Muller et al., 2002) (Figures 5 and 6). This is the "canonical pathway" for recruiting PcG proteins but in vertebrates, it has been shown that the PRC1 can also be recruited independently from H3K27me3 (Morey et al., 2013).

The dRAF complex is composed by the proteins SCE/dRING and PSC, both members of PRC1, and dKDM2. This complex is responsible for two repressive biochemical activities due to the presence of dKDM2: it demethylates dimethylated H3K36 (H3K36me2) and it enhances ubiquitylation of H2A (H2Aub) by SCE/dRING (Figure 6). This complex is considered a "super-repressive" complex as it has been shown to ubiquitylate H2A more efficiently than PRC1 and removes the activating mark H3K36me2 established by the trxG proteins (Lagarou et al., 2008).

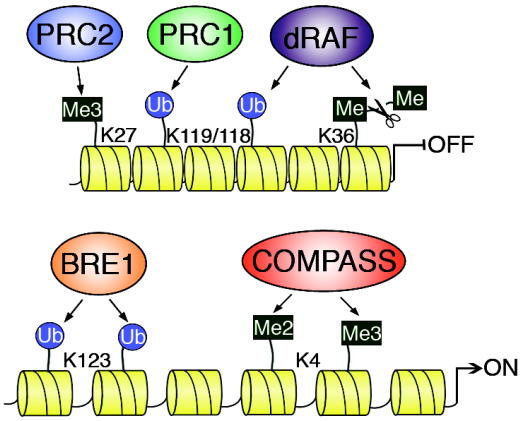


Figure 6. PcG and trxG proteins post-translationally modify histone tails. Simplified representation of the biochemical function of the PcG complexes PRC2, PRC1 and dRAF, and the trxG complexes BRE1 and COMPASS. PRC2 is responsible for trimethylation of H3K27, PRC1 for H2AK119 ubiquitylation (K118 in *Drosophila*) and dRAF for H2Aub and for demethylation of H3K36me2. BRE1 is responsible for H2BK123 ubiquitylation, which recruits the COMPASS macro-complex to di- and trimethylate H3K4.

Among the trxG complexes (Table 3) of interest for this thesis work are the dBRE1/Rad6 complex (Hwang et al., 2003; Robzyk et al., 2000) and the dCOMPASS (Complex of proteins associated with Set1) complexes (Mohan et al., 2011). Although the dBRE1/Rad6 complex has not been described yet as a trxG complex we have included it as a trxG as it post-translationally modifies histones to promote transcriptional activation.

The dBRE1 protein is a RING domain-containing E3 ligase that interacts with the E2 conjugase enzyme Rad6 to ubiquitylate histone H2B in K123 (H2Bub), a modification responsible for transcriptional activation (Nakanishi et al., 2009) (Figures 5 and 6). H2Bub is a pre-requisite for the COMPASS complex to modify H3K4 and therefore promote activation (Davie and Murphy, 1990; Nakanishi et al., 2009). The COMPASS complex is a "macro-

complex" responsible for mono-, di- and trimethylation of H3K4 (Figure 6). In *Drosophila* there are 4 COMPASS complexes: 1) Trithorax (Trx)/COMPASS, 2) Trithorax-related (Trr)/COMPASS, 3) dSet1/COMPASS y 4) Ash1/COMPASS (Herz et al., 2013; Mohan et al., 2011). The crosstalk between H2Bub and H3K4 di- and trimethylation is still not clear, as recent evidence supports it is the interaction between the dBRE1/Rad6 and the dCOMPASS complexes through Wdr82 protein what is required for modifying H3K4 rather than the ubiquitylation of H2B itself (Thornton et al., 2014).

Table 3. Composition of trxG protein complexes

Complex	*Drosophila* Protein	Human homolog	Domain	Biochemical activity
TAC1/COMPASS	Trithorax (TRX)	MLL, MLL2, 3 y 5	SET	H3K4me3 methyl transferase
TAC1	dCBP	CBP	Zn finger, KIX IBiD	Acetyl transferase
TAC1	dUTX	UTX	JmjC	H3K27me3 and me2 demethyl transferase
TAC1/COMPASS	Absent, small o homeotic discs 1 (ASH1)	ASH1L	SET	H3K4/H3K36 methyl transferase
TAC1	Absent, small o homeotic discs 2 (ASH2)	ASH2L, WRD5	Repeticiones WD40	Esencial para H3K4me3
SWI-SNF	Brama (BRM)	BRM, BRG1	Bromodominio, SWI-SNF-like helicasa	Actividad ATPase, Binds HAc
SWI-SNF	Moira (MOR)	SMARCC2, BAF17A		
SWI-SNF	Osa (OSA)	ARID1A, BAF250		
SWI-SNF	Snf5-related 1 (SNR1)	SNF5, ARID4A/B		
COMPASS	dSET1	SET1	SET	H3K4 methyl transferase
COMPASS	Trithorax-related (TRR)		SET	H3K4 methyl transferase
dBRE1-RAD6	dBRE1	BRE1	RING Zn finger	H2B ubiquityl ligase

3.2. The dRYBP gene and protein

dRYBP (Ring1B and Yin yang1 Binding Protein) is a Polycomb-dependent transcriptional repressor that biochemically and genetically interacts with PcG and trxG proteins thus, it is classified as an ETP (Enhancer of Trithorax and Polycomb) (Bejarano et al., 2005; Gonzalez et al., 2008). The *dRYBP* gene is evolutionary conserved and codifies for a nuclear small protein of 17kDa ubiquitously expressed throughout development (Figure 7) (Bejarano et al., 2005). dRYBP contains, in its N-terminal, a Ubiquitin Binding Domain (UBD) of the Nucleoporin Zinc Finger (NZF) type (Bejarano et al., 2005), also philogenetically conserved and that has been shown to interact with ubiquitin (Alam et al., 2004; Arrigoni et al., 2006) (Figure 7).

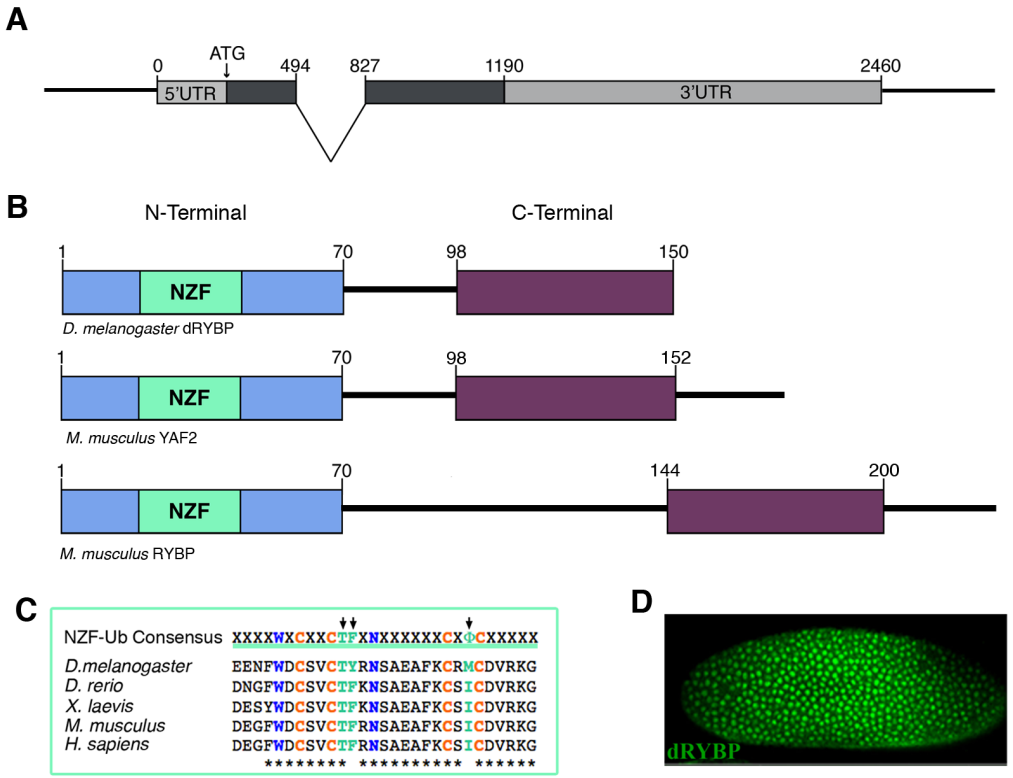


Figure 7. The dRYBP gene and protein. (**A**) The *dRYBP* gene structure. The 5'UTR and 3'UTR regions (grey), the exons (black) and the intron (line) are indicated. (**B**) The dRYBP protein structure. The N-terminal (blue) which contains a NZF motif (green) and the C-terminal (purple) are indicated. Both regions are phylogenetically conserved in murine YAF2 (YY1 associated factor) and in murine RYBP (dRYBP mammalian homolog). (**C**) Sequence of the NZFub domain of dRYBP and the homologous genes in the indicated species. The Cys responsible for Zn binding (orange), the aminoacids responsible for protein folding (blue) and the residues required for ubiquitin binding (green, arrow) are labeled. (**D**) Wild type *Drosophila* embryo showing dRYBP (green) expression. Note dRYBP is expressed in all cell nuclei.

Loss of *dRYBP* function produces a range of phenotypes that are highly variable in penetrance and expressivity including lethality, female sterility, embryonic mitotic collapse and organ size reduction (Gonzalez et al., 2008), suggesting that dRYBP participates in an array of biological processes. Furthermore, loss of *dRYBP* function does not produce homeotic phenotypes, but in a sensitized mutant genetic background, for example in an heterozygous *Sce/dRing* mutant or in an heterozygous *trx* mutant, loss of *dRYBP* function enhances the homeotic phenotypes related to *Sce/dRing* and *trx* mutants, confirming once again that the *dRYBP* gene functions as an ETP (Bejarano et al., 2005; Gonzalez et al., 2008).

Moreover, high levels of dRYBP induce apoptosis (Gonzalez and Busturia, 2009) and interestingly, high levels of human RYBP induce apoptosis only in transformed cells (Novak and Phillips, 2008). In addition, both the fly and the vertebrate RYBP interact with a variety of apoptosis-related proteins, among them FADD and DREDD, the apoptotic related factors containing DD (Death Domain) and DED (Death Effector Domain) (Gonzalez and Busturia, 2009; Zheng et al., 2001), Apoptin, a viral apoptosis agonist (Danen-van Oorschot et al.,

2004), Hippi, a pro-apoptotic protein first described in the context of the Huntington's disease (Stanton et al., 2007), and MDM2, an E3 ubiquitin ligase which negatively regulates p53 (Chen et al., 2009). In spite of this, the molecular mechanisms controlling dRYBP/RYBP-mediated apoptosis are poorly understood.

4. THE APOPTOTIC PATHWAY

Apoptosis is a process that occurs during normal development to assure correct morphogenesis and during the adult life to eliminate cells with damaged DNA or chromosome abnormalities. Moreover, apoptosis helps maintaining organism homeostasis as it is activated when cellular transformation takes place, in order to eliminate potentially malignant cells (Conradt, 2009; Favaloro et al., 2012; Fuchs and Steller, 2011). The pathways controlling apoptosis are highly conserved from flies to humans (Steller, 1995). Cell death machinery mainly consists of a family of proteases, caspases, activated by intrinsic and/or extrinsic signals (MacKenzie and Clark, 2012). In *Drosophila*, as well as in mammals, the intrinsic pathway is stimulated by intracellular death-inducing signals that activate pro-apoptotic proteins, including Reaper, Hid and Grim (RHG) (Wing et al., 2001), which function is to inhibit DIAP1 (*Drosophila* Inhibitor of Apoptosis Protein 1) (Hay et al., 1995). Consequently, DIAP1 cannot inhibit initiator caspase Dronc, which then binds to Ark (Apaf-1 related killer) forming the apoptosome and, ultimately, promoting cell death via the activation of the effector caspases Drice and Dcp-1 (Fuchs and Steller, 2011; MacKenzie and Clark, 2012; Steller, 1995) (Figure 8).

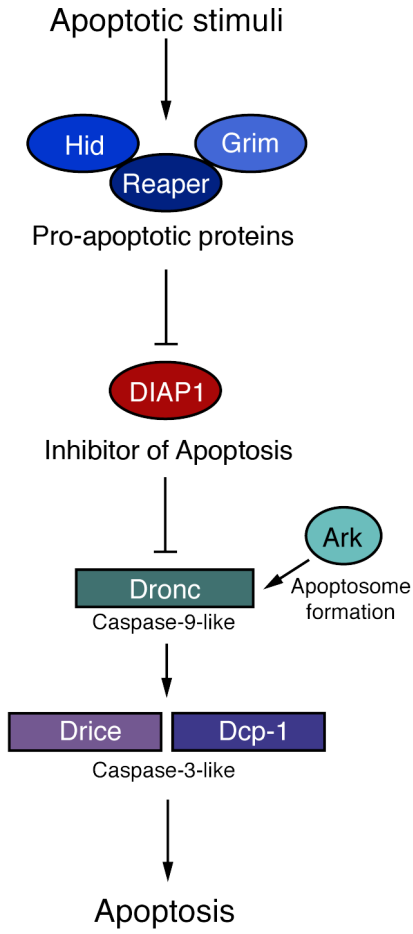


Figure 8. The intrinsic apoptotic pathway. The apoptotic pathway remains latent during development and under an apoptotic stimuli expression of pro-apoptotic genes *rpr*, *hid* and *grim* is induced. The function of pro-apoptotic proteins is to bind to DIAP1 promoting its destruction. This translates into the activation of initiator caspase, Dronc that binds to Ark to form the apoptosome, leading to the activation of effector caspases Drice and Dcp-1 to promote cell death.

The control of both the activation and repression of the apoptotic cascade is essential for the survival of the organism. For example, poor activation leads to defects in morphogenesis and cancer while prolonged activation can be the cause of several neurodegenerative diseases (Favaloro et al., 2012). From the death stimuli to the activation of the effector caspases, the regulation of the apoptotic pathway includes precise transcriptional and post-translational control to assure its correct outcome (Conradt, 2009; Fuchs and Steller, 2011). Protein ubiquitylation functions to regulate the abundance of apoptotic proteins and, thereby, to control both activation and repression of the apoptotic cascade (Bergmann, 2010; Vucic et al., 2011). In the control of the *Drosophila* intrinsic cell death pathway, it has been shown that the RHG proteins can be ubiquitylated by DIAP1 protein promoting their degradation by the proteasome system(Olson et al., 2003). Moreover, it has been shown that the RHG proteins bind to DIAP1, a RING domain containing protein, stimulating DIAP1 autoubiquitylation and degradation and thereby, facilitating cell death (Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). Therefore, the levels of pro-apoptotic proteins and DIAP1 are essential to maintain the balance between cell death and cell survival. Moreover, other proteins in the intrinsic apoptotic pathway, such as caspases Dronc and Drice, have been shown to be ubiquitylated, but the function of this post-translational modification still remains unclear.

OBJECTIVES

The objectives of this thesis work were to study the functionality of the dRYBP protein, using *Drosophila melanogaster* as a model system, in two different biological processes where protein ubiquitylation plays an important role: epigenetic regulation of gene expression mediated by PcG and trxG proteins, and the regulation of the apoptotic pathway. Both of these mechanisms of gene regulation are crucial for cellular homeostasis and therefore, to understand normal and pathological development.

The specific objectives of this thesis work were:

1. Analysis of dRYBP molecular function

2. Functional study of dRYBP in the epigenetic regulation of gene expression

3. Functional study of dRYBP in apoptosis regulation

Experimental Procedures

1. FLY STRAINS AND GENETICS

Fly strains containing mutant alleles used in this work were: *dRYBP*^{1}(Gonzalez et al., 2008), *Sce*^{1}(Breen and Duncan, 1986), *Pc*^{3}(Breen and Duncan, 1986), *dBre1*^{kim1} (van der Knaap et al., 2010) and *dkdm2*^{KG04325} (described in www.flybase.org).

The GAL4/UAS system was used for overexpression and inactivation experiments (Brand and Perrimon, 1993). The GAL4 and UAS lines used in this work were: *engrailed-Gal4 (enGal4), scalloped-Gal4 (sdGal4), rotund-Gal4 (rnGal4), Distal-less-Gal4 (DllGal4), apterous-Gal4 (apGal4), hedgehog-Gal4 (hhGal4),* (described in www.flybase.org), *UAS-rpr-HA* (Ryoo et al., 2002), *UAS-dRYBP* (Gonzalez and Busturia, 2009), *UAS-slimb, UAS-pall, UAS-DIAP1, UAS-p35, UAS-GFP, UAS-p53* (described in www.flybase.org), *UAS-skpA*$_{RNAi}$, *UAS-slimb*$_{RNAi}$, *UAS-p53*$_{RNAi}$ (Transgenic RNAi Project, www.flyrnai.org/TRiP-HOME.html), *UAS-dCul1*$_{RNAi}$, *UAS-pall*$_{RNAi}$, *UAS-rpr*$_{RNAi}$ (Dietzl et al., 2007).

Generation of transgenic lines: The *UAS-skpA* and *UAS-dCul1* transgenic lines used in this study were generated by injection of the pUASt-skpA and pUASt-dCul1 DNA constructs kindly provided by P.H. O'Farrell, University of California, San Francisco, described in (Heriche et al., 2003) following standard *Drosophila* transgenic procedures and using *w*^{1118} flies as host (Rubin and Spradling, 1982). Resulting flies were crossed with *w*^{1118} to identify transgenic flies and the stock *Df(1)w*^{67c23}*;If/CyO;MKRS/TM6B* to localize the insertion in the different chromosome and to balance the stocks. To differentiate previous *UAS-skpA* and *UAS-dCul1* transgenic lines from the ones used in this study, we named these lines *UAS-skpA*^{OB} *and UAS-dCul1*^{OB}.

Transgenic flies containing the *reaper-4kb-lacZ* (*rpr-lacZ*) (Jiang et al., 2000), *puckered-lacZ (puc-lacZ)* (Martin-Blanco et al., 1998) and *diap1-lacZ* (Hay et al., 1995) elements were used as reporters for *rpr*, *puc* and *diap1* expression respectively.

Flies were grown in standard fly media and fly crosses were maintained either at 17ºC, 25ºC or 29ºC depending on the specific experimental requirements.

2. CELL CULTURES

Drosophila S2 and S2R+ cells were grown in standard conditions at 27ºC in Cell Growth Media: Schneider's Drosophila Medium + 10% Fetal Bovine Serum (Life Technologies), 50U/ml Penicillin and 50µg/ml Streptomycin.

Human embryonic kidney 293 (HEK293) cells were grown in standard conditions at 37ºC in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% fetal Bovine Serum (Life Technologies), 100 U/ml Penicillin and 100 mg/ml Streptomycin.

3. GENERATION OF DNA CONSTRUCTS

The plasmid pUASt-dRYBP (Gonzalez and Busturia, 2009) was used as template to amplify dRYBP and dRYBPΔNZF cDNAs by PCR reaction using the primers indicated in Table 4. Next, dRYBP and dRYBPΔNZF were cloned into NdeI/XbaI pGEX-2TKN vector (Pharmacia).

The plasmid pUASt-skpA was used as a template to amplify skpA cDNA by PCR reaction using the primers indicated in Table 4 and to add the FLAG-tag. skpA-FLAG tag was cloned into a BamHI/NotI pCDNA-3.1 vector (Sandu et al., 2010).

The digestion using NdeI and XbaI or BamH1 and NotI, ligation and transformation were done following standard procedures (Sambrook, 1989) (See Table 5 for references).

Table 4. Primers used for cloning

Primers	Sequence
dRYBP Fw	TTTCATATGATGGACAAGAAATCCTCGCCG
dRYBP Rv	TTTTCTAGACTAACTCCGGCTGTCGTTGCT
dRYBPΔNZF Fw	TTTCATATGGCCTCCGGATCACGGCATGGC
dRYBPΔNZF Rv	TTTTCTAGACTAACTCCGGCTGTCGTTGCT
skpAFLAG Fw	CAAGGGATCCATGCCCAGCATCAAG
skpAFLAG Rv	GCTTGCGGCCGCCTTGTCGTCATCGTCTTTGTAGTCCTTCTCCTCGCACCAC

The resulting plasmids were sequenced after cloning to confirm the sequence and orientation of the gene of interest at the sequence facilities at the Erasmus Medical Center, Rotterdam and The Rockefeller University, New York.

Table 5. Enzymes used for cloning

Enzyme	Reference
Adv Taq Polimerase	Sigma
NdeI	NE Biolabs
XbaI	NE Biolabs
BamHI	NE Biolabs
NotI	NE Biolabs
T4 Ligase	Roche

4. CELL TRANSFECTION

Transfection was done with FuGENE 6 (Roche) according to the manufacturer's instructions. 48h after transfection, the cells were lysed and analyzed for protein expression or used as input for anti-FLAG IP. skpAFLAG construct was generated for this thesis work and Rpr-HA and DIAP-GST (Sandu et al., 2010) vectors were a gift from Dr. Hermann Steller, The Rockefeller University, New York.

5. dsRNA GENERATION AND RNAi-MEDIATED KNOCKDOWNS (KD)

pUASt-dRYBP and pUASt-skpA were used as template for PCR reaction using specific primers to generate double stranded RNA (dsRNA) (Table 6). dsRNA was synthesized using

the MEGAscript T7 kit (Ambion). Then dsRNA was precipitated with 3M Sodium Acetate + 100% ethanol o/n at -20ºC. The following day, dsRNA was spinned down at maximum speed for 15min at 4ºC, the ethanol was removed and pellets were resuspended in 50µl of RNAse free H_2O. For inactivation in S2/S2R+ cells, 1 million of cells were resuspended in Serum-free media and 15µg of corresponding dsRNA were added (GFP dsRNAi was used for the Mock). Samples were incubated for 60min at RT and then Schneider's complete medium was added. Cells were returned to 27ºC and after 4 days samples were collected for whole protein or acid histone extraction.

Table 6. Primers used for dsRNA generation

Primers	Sequence
dRYBP Fw_1	5'-TTAATACGACTCACTATAGGGAGAGACAAGAAATCCTCGCCGGT-3'
dRYBP Rv	5'-TTAATACGACTCACTATAGGGAGAGTGATCGAGGAGAACTTCTGG-3'
dRYBP Fw_2	5'-TTAATACGACTCACTATAGGGAGAGCTGTCGTTGCTCTCGCTGAA-3'
skpA Fw_1	5'-TTAATACGACTCACTATAGGGAGACCCAGCATCAAGTTGCAATCT-3'
skpA Rv	5'-TTAATACGACTCACTATAGGGAGACTTCTCCTCGCACCACTCGTT-3'

dsRNAs used for inactivation of dRING, dKDM2, PC (Lagarou et al., 2008) dBRE1 (van der Knaap et al., 2010) were a gift from Prof. C. Peter Verrijzer, Erasmus Medical Center, Rotterdam.

6. PROTEIN EXTRACTION

Cells were harvested by scraping with a cell lifter to dislodge cells from the dish and transferred directly into 1.5ml tubes. Then, samples were centrifuged at 1000rpm for 5min and washed with PBS. Cells were lysed by resuspension with 200µl of RIPA Lysis Buffer (150mM NaCl + 1%Nonidet P-40, 0.5% Deoxycholate + 1%SDS + 50mM Tris-HCl pH 8 + 10mM NaF + 0.4mM EDTA pH 8.0, 10% Glycerol) and pushed through a needle 3 times to ensure lysis. Next, samples were centrifuged at maximum speed for 30min at 4ºC to pellet cell debris and supernatant was store at -20ºC until use.

7. ACID EXTRACTION OF CORE HISTONES

Cells were harvested and washed following the same protocol as for protein extracts (Section 6, Experimental Procedures). In this case, cells were lysed with lysis buffer (10mM Tris-HCl pH 7.5 + 1mM $MgCl_2$ + 0.5% NP-40 + minitablet of Protease inhibitors, Roche + 0.1mM DTT and 0.2mM PMSF) for 10min on ice and pushed through a syringe 3 times. Next, samples were

centrifuged at 10000rpm for 1min and the pellet was washed with wash buffer (10mM Tris-HCl pH 7.5 + 1mM $MgCl_2$ + 400mM KCl, minitablet of Protease inhibitors, Roche) once. Then, histone extraction was performed by resuspending pellet with 50ml of extraction buffer (10mM Tris HCl pH 7.5 + 20% Glycerol + 0.4M HCl) for 10min on ice. Samples were centrifuged at 10000rpm for 10min and the last step was repeated with half volume of extraction buffer. After that, the supernatants were pulled together and incubated with acetone o/n at-20ºC. Finally, samples were spinned down, washed 3 times with acetone and pellet was resuspended in 100ml of 2X SDS-PAGE loading buffer. Samples were stored at -20ºC until protein detection or pulldown assay.

8. PROTEIN EXPRESSION AND PURIFICATION

pGEX-dRYBP-GST and pGEX-dRYBPΔNZF-GST were transformed in competent *E. Coli* strain BL21 and colonies were allowed to grow o/n.

BL21 single colonies were inoculated into 10ml of LB + 50mg/ml Amp and grown o/n at 37ºC. The next day 5ml of the culture were transferred into a 500ml LB + 50mg/ml Amp + 10mM $ZnCl_2$. Bacteria were grown until OD_{600}=0.5 and then IPTG was added to a final concentration of 0.2mM and grown at 25ºC for 3h. Next, the cells were centrifuged at 4000rpm for 20min, the pellet was washed with 10ml of PBS and centrifuged again. To lyse the cells the pellet was resuspended in 10ml of lysis buffer (25mM HEPES pH 7.6 + 10% Glycerol + 0.5M NaCl + 0.01% NP40 + 3mM DTT + minitablet of Protease inhibitors, Roche+ 50uM $ZnCl_2$ + 0.2mM PMSF) + 0.015mg/ml lysozyme and sonicated 30s ON/OFF with 80% Amplitud for 8min. Next, cells were spined at 17000rpm for 30min and supernatant was stored at -20ºC until use.

For protein purification, 500µl of Glutathione Sepharose 4B beads were added per 5ml of lysed supernatant. Then, samples were mixed at 4ºC for 2.5h and washed 3 times for 5min with wash buffer (10mM Tris pH 7.5 + 1mM EDTA + 100mM NaCl + 0.2% NP40).

For GST-pulldowns, protein purification protocol was stopped at this point.

For antibody generation, proteins were eluted by adding 100µl of elution buffer (1xHEMG + 0.5M NaCl + 0.01% NP40 + 10mM reduced glutathione) to the beads and then, samples were mixed at 4ºC for 1h. Finally, eluted samples were stored at -80ºC until sent for antibody generation to the Erasmus Medical Center Facility, Rotterdam.

9. PROTEASOME INHIBITION

S2 cells were incubated with the proteasome inhibitors 5µM Lactacystin (Millipore) and 50µM MG-132 (Millipore) or treated with DMSO (Mock) as a control. Cells were collected 12h later and proteins were extracted following previously described protocols.

Protein extracts in these conditions were used for GST-pulldown assay and for direct protein detection by Western Blot (WB).

10. IMMUNOPRECIPITATION

10.1. Crossed-linked antibody IP

Previous to immunoprecipitation, dRYBP antibody was crossed-linked to Sepharose A beads (GE Healthcare). For that end, 1ml of dRYBP antibody (Bejarano et al., 2005) was incubated with 200µl of Sepharose A beads for 30min at RT. The sample was centrifuged at 1000rpm for 3min to pellet the beads and the supernatant was discarded. Next, 0.005g of dimethylpimelidate (Sigma-Aldrich) were added in 1ml of 0.2M Sodium Borate pH 9.0 and mixed for 30min at RT to cross-link the antibody to the beads. To stop the reaction, the beads were centrifuged at 1000rpm for 3min and incubated with 1ml of 0.2M Ethanolamine pH 8.0 for 1h at RT. Finally the beads were washed twice with 1M PBS and stored at 4ºC until used for IPs.

For immunoprecipitation, dNE (*Drosophila* embryonic nuclear protein extracts, a gift from C. Peter Verrijzer, Erasmus Medical Center, Rotterdam) were incubated with anti-dRYBP antibody coupled to Sepharose A beads and Sepharose A beads (non-coupled to antibody) used as Mock. The samples were incubating by rotating for 2h at 4ºC and centrifuged at 1000rpm for 3min to pellet the beads. Then, the beads were washed twice with HEMG buffer (50mM HEPES + 0.2mM EDTA + 25mM $MgCl_2$ + 20% glycerol) containing 200mM KCl, followed by 1 wash with HEMG containing 400mM KCl. After each wash the tube was centrifuged at 1000rpm for 3min to pellet the beads and the supernatant was discarded. Finally, elution buffer (100mM Glycine + 150mM NaCl, pH 2.5) was added to the beads and then centrifuged at 1000rpm for 5min. The supernatants containing immunoprecipitated proteins were neutralized with Tris pH 9.0 and stored at -20ºC.

10.2. Non crossed-linked antibody IP

500µl of dNE were incubated with 100µl of the corresponding antibody or with pre-immune serum used as Mock o/n at 4ºC. The following day 100µl of Sepharose A beads (GE Healthcare) was added to each sample and mixed for 2h at 4ºC. Then, samples were washed

following the previously described procedures. Finally, 100µl of 6x SDS-PAGE loading buffer was added and proteins were eluted from beads by boiling samples at 95ºC for 5min. Eluted proteins were stored at -20ºC.

10.3. anti-FLAG tag IP

200µl of lysed HEK293 cells were incubated with 30µl of beads coupled to anti-FLAG antibody (Sigma) for 2h at 4ºC. Non transfected HEK293 cells were used as negative control for the IP. Then, samples were washed 3 times for 10min at 4ºC with the previously described wash buffer. Finally, 30µl of 6x SDS-PAGE loading buffer was added and proteins were eluted from beads by boiling samples at 95ºC for 5min. Eluted proteins were stored at -20ºC.

11. GST-PULLDOWN ASSAY

100µl of dRYBP-GST, dRYBPΔNZF-GST previously coupled to GST-Sepharose beads (GE Healthcare) or GST-Beads (used as Mock) were incubated with 300µl of dNE, S2 cells protein extracts or histone extracts, depending on the experimental design, for 2h at 4ºC. Samples were washed 3 times with 10 volumes of washing buffer (10mM Tris pH 7.5 + 1mM EDTA + 0.2% NP40 + 150mM NaCl) and proteins were eluted from beads by adding 200µl of 6xSDS-PAGE loading buffer and boiling samples for 5min at 95ºC. Eluted proteins were stored at -20ºC.

12. WESTERN BLOT ANALYSIS

Protein samples were separated by an 8, 12, 15 or 18% SDS-PAGE gel, depending on the MW of the protein to be analyzed, and then transferred to a nitrocellulose membrane (Whatman) following standard procedures. The membrane was incubated with blocking buffer (PBS containing 3% BSA) at RT for 30min and rinsed with PBT buffer (PBS containing 0.05% Tween-20). Next, the membrane was incubated o/n at 4ºC with the corresponding primary antibody (Table 7). Subsequently, the membrane was washed 3 times with PBT for 10min at RT and incubated with a secondary antibody for 1h at RT and then, washed again. Secondary antibodies (Table 8) coupled to HRP (Horse Radish Peroxidase) or to AP (Alkaline Phosphatase). Finally, protein detection was carried out using an ECL (Enhanced Chemiluminiscence) reagent detection kit (GE Healthcare Life Sciences) for HRP-coupled secondary antibodies and for those coupled to AP, membranes were incubated with NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-choloro-3-indolylphosphate) (Thermo Scientific) until protein was detected. To stop the developing reaction membranes were washed with distilled H_2O.

Table 7. Primary antibodies used for WB

Antibody	Dilution	Raised in	Reference
Anti -dRYBP	1:250	Rabbit	(Bejarano et al., 2005)
Anti-SKPA	1:1000	Rabbit	(Hughes et al., 2008)
Anti-dCUL1	1:1000	Rabbit	Invitrogen
Anti-H2A	1:500	Rabbit	Abcam 13293
Anti-H2Aub	1:250	Mouse	Merck Millipore clone E6C5
Anti-H2B	1:2000	Rabbit	Upstate (Millipore 07-371)
Anti-H2Bub	1:1000	Mouse	Millipore clone 56
Anti-H3	1:2000	Rabbit	Abcam 1791
Anti-H3K36me2	1:2000	Rabbit	Upstate (Millipore 07369)
Anti-H3K27me3	1:1000	Rabbit	Upstate 07-449
Anti-H3K4me3	1:1000	Rabbit	Active Motif 39159
Anti-H3K4me	1:500	Rabbit	Abcam ab8895
Anti-ub (FK2)	1:2000	Mouse	Millipore
Anti-tubulin	1:8000	Mouse	Sigma
Anti-PC	1:500	Rabbit	(Lagarou et al., 2008)
Anti-PSC	1:250	Guinea pig	(Lagarou et al., 2008)
Anti-dRING	1:500	Guinea pig	(Lagarou et al., 2008)
Anti-dKDM2	1:250	Guinea pig	(Lagarou et al., 2008)
Anti-dBRE1	1:1000	Rabbit	(van der Knaap et al., 2010)
Anti-PH	1:500	Rabbit	(Lagarou et al., 2008)
Anti-EZ	1:250	Guinea Pig	(Lagarou et al., 2008)
Anti-DIAP1	1:1000	Rabbit	(Ryoo et al., 2002)
Anti-p53	1:1000	Mouse	Iowa Hibrydoma Bank
Anti-rpr	1:1000	Chicken	(a gift from H. Steller, unpublished)

Table 8. Secondary antibodies used for WB

Antibody	Dilution	Raised in	Reference
Anti-mouse-HRP	1:30000	Rabbit	Sigma
Anti-rabbit -HRP	1:5000	Donkey	GE Healthcare
Anti-chicken-HRP	1:10000	Rabbit	Promega
Anti-rabbit-AP	1:10000	Goat	Sigma
Anti-Guinea pig-AP	1:10000	Goat	Sigma

13. X-GAL STAINING

Imaginal discs were dissected from 3rd instar larvae in PBS solution and fixed in PBS + 0.5% Glutaraldehyde solution for 10min on ice. Then, imaginal discs were washed 3 times with wash buffer (PBS + 0,1% Triton) for 10min. Finally, imaginal discs were incubated in 1ml of developing solution (10mM NaH_2PO_4/Na_2HPO_4 + 150mM NaCl + 1mM $MgCl_2$ + 3.5mM K_4FeCN_6, pH 7.4) and 30% X-GAL (Promega). Samples were incubated at 37ºC until they developed blue staining and then were mounted in 10% glycerol for subsequent analysis.

14. IMMUNO STAINING

Imaginal discs were dissected from 3rd instar larvae in PBS and fixed in a solution containing 4% Paraformaldehyde + 0,1% Triton and 0,1% DOC for 30min at RT. Samples were then washed 3 times with wash buffer (PBS + 0,1% Triton) each for 10min and blocked with blocking buffer (1% BSA in PBS) for 30min at RT. Next, samples were stained with the corresponding primary antibody in blocking solution o/n at 4ºC. The following day samples were washed 3 times with wash buffer for 10min and incubated with the corresponding secondary antibody coupled to a fluorophore in blocking solution for 1h at RT. To-pro-3 was incubated for 10min for DNA staining. Finally, samples were washed 3 times for 10min and mounted in Vectashield (Vector Laboratories) for subsequent analysis. Tables 9 and 10 show antibodies used for immunostaining analysis.

Table 9. Primary antibodies used for immunostaining

Antibody	Dilution	Raised in	Reference
Anti-activated Caspase 3 (C3)	1:500	Rabbit	Cell Signaling Technologies
Anti-SKPA	1:500	Rabbit	(Hughes et al., 2008)
Anti-β GALACTOSIDASE (β-GAL)	1:100	Mouse	Promega
Anti-DIAP1	1:500	Rabbit	(Ryoo et al., 2002)
Anti-UBX	1:20	Mouse	Iowa Hibrydoma Bank

Table 10. Secondary antibodies used for immunostaining

Antibody	Dilution	Raised in	Reference
Anti-mouse Alexa 488	1:500	Donkey	Invitrogen
Anti-rabbit Alexa 488	1:500	Rabbit	Invitrogen
Anti-rabbit Alexa 555	1:500	Rabbit	Invitrogen
To-pro-3	1:500		Invitrogen

15. FLUORESCENCE AND X-GAL QUANTIFICATION AND STATISTICAL ANALYSIS

Wing imaginal discs were stained following previously described procedures (Section 14 Experimental Procedures) and photographed using a LSM510 confocal microscope. The wing disc area designated for fluorescence quantification was determined by the GFP expression domain driven by either the *sdGal4*, *hhGal4* or *enGal4* transgenic lines. The leg disc area designated for X-GAL quantification was determined by the GFP expression domain driven by the *rnGal4* transgenic line. Fluorescence and X-GAL staining was measured and quantified using Fiji imaging software calculating the different Fluorescence/Area ratios. Statistical analysis was performed using Wilcoxon paired data test using the GraphPad Prism software. Data represents the mean + SEM (Standard Error of the Mean).

16. IRRADIATION OF IMAGINAL DISCS

Early 3rd instar larvae grown at 29ºC were irradiated at a dose of 2000R using a Philips X-ray instrument and then returned to 29ºC. Twenty-four hours after irradiation, the larvae were dissected and immunostained with anti-activated C3 antibody following previously described procedures (14).

17. SCANNING ELECTRON MICROSCOPY

Adult legs were dissected from 1 to 2 day-old flies and processed following standard procedures at the scanning electron microscopy facility of the Universidad Autónoma de Madrid.

18. CUTICLE PREPARATION

For preparation of larval cuticle, the larvae were washed with water, mounted in Hoyer and incubated o/n at 60ºC.

For preparation of adult cuticles, the flies were dissected in water and incubated for 10min in 10% KOH at 90ºC in order to eliminate the fat. Next, cuticles were washed with water, dehydrated with 100% EtOH and mounted in Euparal (ANSCO).

19. MICROSCOPY

Immunofluorescence images were generated in a LSM510 confocal microscope and X-GAL staining and cuticles images were generated in a Zeiss CCD microscope. Images were subsequently processed using Adobe Photoshop CS5 and Fiji imaging software for quantification.

20. SURVIVAL ASSAY

First instar larvae were collected from egg laying plates, transferred into food vials and placed at 25°C. Survival was monitored over developmental time.

21. QUANTITATIVE RT-PCR

RNA was isolated from S2R+ cells and extracted using RNA isolation kit (Ambion). RT-qPCR was performed using SYBR Green One-Step (Thermo-Scientific) in an Applied Biosystems 7900 Sequence Detector System. Quantified mRNA levels were expressed as relative fold change normalized to RP49. Primer sequences are indicated in Table 11.

Table 11. Primers used for RT-qPCR

Gene	Sequence
RP49 Fw	5' TACAGGCCCAAGATCGTGAAG 3'
RP49 Rv	5' GACGCACTCTGTTGTCGATACC 3'
dRYBP Fw	5' CATGTTGACACCTGGCTCCTG 3'
dRYBP Rv	5'CGAAGGTGATCGAGGAGAAC 3'
skpA Fw	5' CTCCCGAGGAAATACGCAAG 3'
skpA Fw	5' CGGGCGAAAAGTCCTTCTTA 3'
Rpr Fw	5' CCAGTTGTGTAATTCCGAACGA 3'
Rpr Rv	5' GGATCTGCTGCTCCTTCTGC 3'
Hid Fw	5' CATCAGTCAGCAGCGACAGG 3'
Hid Rv	5' ACGAAAACGGTCACAACAGTTG 3'

Results

1. ANALYSIS OF dRYBP MOLECULAR FUNCTION

The dRYBP protein (MW=17kDa) is evolutionary conserved (Bejarano et al., 2005) (Figure 7) and contains a Ubiquitin Binding Domain (UBD) of the Nucleoporin Zinc Finger (NZF) type in its N-terminal (Alam et al., 2004; Bejarano et al., 2005). The NZF is a zinc-binding domain composed of 35 aminoacids present in several proteins involved in the ubiquitin pathway (Alam et al., 2004; Hicke et al., 2005; Wang et al., 2003). The NZF found in the dRYBP protein is also evolutionary conserved, and its mammalian homolog RYBP has been shown to be ubiquitylated and to interact with ubiquitylated proteins (Alam et al., 2004; Arrigoni et al., 2006) (Figure 7).

To investigate the functionality of the UBD domain of the dRYBP protein we studied whether dRYBP could bind ubiquitin and also if dRYBP was capable of interacting with ubiquitylated proteins.

1.1. The dRYBP protein is monoubiquitylated

To analyze the functionality of the UBD domain of dRYBP, protein extracts from *Drosophila* S2 cells untreated (Mock) and treated with the proteasome inhibitors MG-132 and Lactacystin, were resolved by SDS-PAGE and analyzed by WB with both anti-dRYBP (Bejarano et al., 2005) and anti-ubiquitin antibodies (Table 7). Figure 9A shows that the dRYBP antibody (left panel) detects two different bands of two different MW, indicating that the dRYBP protein exists in two different forms, one of 17kDa and one of 25kDa. When comparing the WB showing dRYBP expression (Figure 9A, left panel) to the one showing Ub expression (Figure 9A, right panel) the band of 25kDa is detected with both antibodies. Taking in account that the Ub MW is 8kDa, our results suggest that dRYBP is monoubiquitylated (dRYBPub). Moreover Figure 9A also shows that both in the absence and presence of proteasome inhibitors the bands corresponding to dRYBPub show similar levels of dRYBP and Ub expression, indicating that levels of monoubiquitylation of dRYBP do not vary when the proteasome is inhibited. Therefore, dRYBP monoubiquitylation is not required for its proteasomal degradation.

Thus, these results indicate that dRYBP exists *in vivo* in two forms: dRYBP and dRYBPub.

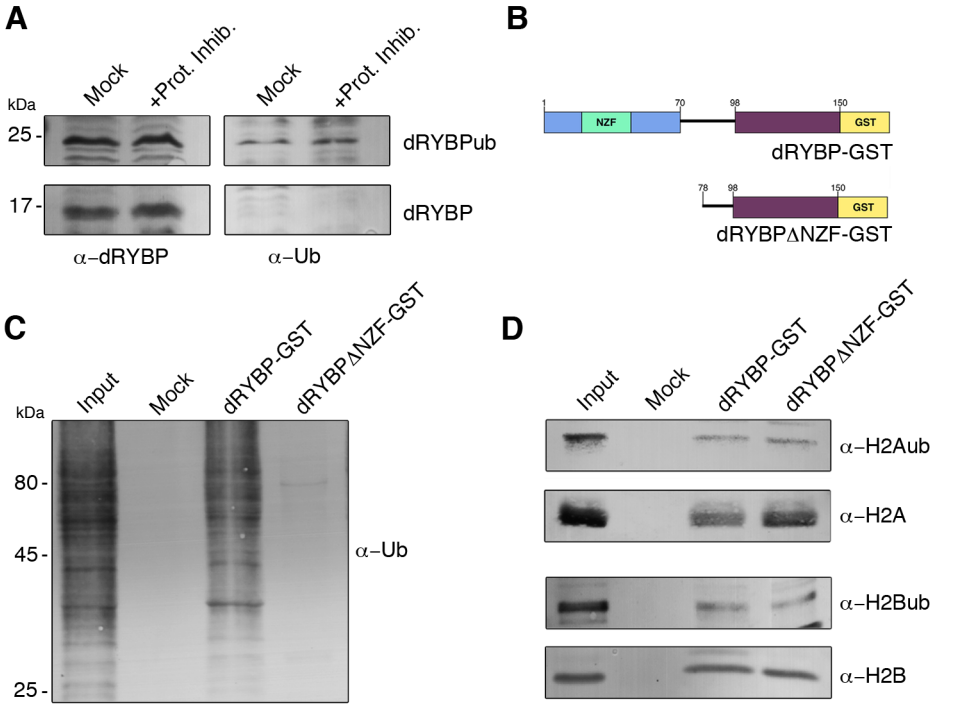


Figure 9. dRYBP binds to ubiquitin, ubiquitylated proteins, H2A, H2B, H2Aub and H2Bub. (**A**) Immunobloting with anti-dRYBP (α-dRYBP) and anti-ubiquitin (α-Ub) antibodies of S2 cells protein extracts of untreated S2 cells (Mock) and treated with the proteasome inhibitors MG132 and Lacacystin (+Prot. Inhib.). Note that α-dRYBP detects two bands, 17kDa and 25kDa, and α-Ub detects the 25kDa band. (**B**) Scheme of the fusion proteins generated for GST-pulldown assays, dRYBP full length (dRYBP-GST) and a truncated version lacking the UBD (dRYBPΔNZF-GST). (**C**) GST-pulldown assay using S2 cell extracts treated with proteasome inhibitors (Input) incubated with GST beads (Mock) or with the fusion proteins dRYBP-GST or dRYBPΔNZF-GST. Eluted proteins were analyzed by WB using α-Ub antibody. Note a great amount of ubiquitylated proteins that bind to full length dRYBP-GST protein do not bind to dRYBPΔNZF-GST protein. (**D**) GST-pulldown assays using S2 cells histone extracts (Input) incubated with GST beads (Mock) or with the fusion proteins dRYBP-GST or dRYBPΔNZF-GST. Eluted proteins were analyzed by WB using the indicated antibodies. Note that dRYBP-GST and dRYBPΔNZF-GST bind to the histones H2A, H2Aub, H2B and H2Bub.

1.2. The dRYBP protein interacts with ubiquitylated proteins

To further understand the molecular functions of dRYBP and its UBD domain, we examined if the dRYBP protein could interact with ubiquitylated proteins and if so, wether it was done through its UBD domain. For that, we generated the constructs dRYBP-GST and dRYBPΔNZF-GST for a pulldown assay (Figure 9B). The dRYBP-GST construct generates the full-length dRYBP protein while dRYBPΔNZF-GST is a truncated form lacking the NZF, which contains the predicted UBD (Gonzalez et al., 2008). The pulldown assay was performed using S2 cells treated with the proteasome inhibitors (Figure 9C) in order to increase the amount of ubiquitylated proteins in cell protein extracts (to observe the low amount of ubiquitylated proteins in untreated with proteasome inhibitor cell protein extracts see Appendix 1). Next, proteins were analyzed by WB, using an anti-ubiquitin antibody that exclusively detects linked

ubiquitin (mono- or poly-) chains but not free ubiquitin (Figure 9C). The results show that full-length dRYBP-GST protein interacts with ubiquitylated proteins (Figure 9C). However, dRYBPΔNZF-GST is not able to bind as many ubiquitylated proteins (Figure 9C) as the full-length dRYBP-GST protein, suggesting that the NZF domain, which contains the UBD, is required for dRYBP to bind a significant amount of ubiquitylated proteins present in S2 cells.

Taken together, our results suggest that dRYBP can bind ubiquitin. Moreover, our results indicate that dRYBP can bind ubiquitylated proteins through its NZF domain and suggest that it may be involved in biological processes where the ubiquitylation is in involved their regulation.

1.3. The dRYBP protein interacts with histones H2A, H2B, H2Aub, H2Bub

Central to epigenetic regulation of gene expression is histone monoubiquitylation (Goldknopf et al., 1975; Zhang, 2003). In particular, ubiquitylation of histone H2A is responsible for gene silencing (Endoh et al., 2012; Wang et al., 2004) and ubiquitylation of histone H2B is responsible for gene activation (Davie and Murphy, 1990; Henry et al., 2003; Osley, 2004). As shown in this thesis work, dRYBP is capable of interacting with ubiquitylated proteins (Figure 9C), so to begin to decipher the role of dRYBP in histone post-translational modifications we sought to study if dRYBP protein could bind to the ubiquitylated histones H2A and H2B (H2Aub and H2Bub) and if the UBD domain was involved in this interaction.

We carried out GST-pulldowns with the full-length dRYBP-GST and dRYBPΔNZF-GST fusion proteins generated for this work (Figure 9B). However, in this case, an acid extraction of core histones from S2 cells was used as input for the pulldown. Eluted proteins were resolved by SDS-PAGE and analyzed by WB with the corresponding antibodies. Figure 9D shows that both full-length dRYBP-GST and dRYBPΔNZF-GST bind to H2A and H2B and the modified variants H2Aub and H2Bub.

Taken together, the pulldown experiments show an interaction between dRYBP protein and histones H2A and H2B as well as to their ubiquitylated form, H2Aub and H2Bub. Moreover, the NZF domain is not necessary for this interaction, suggesting the existence of a different protein domain, required for H2A, H2Aub, H2B and H2Bub binding.

2. FUNCTIONAL STUDY OF dRYBP IN THE EPIGENETIC REGULATION OF GENE EXPRESSION

It has been previously described that the dRYBP protein is able to interact with PcG as well as with trxG proteins (Bejarano et al., 2005; Gonzalez et al., 2008). Moreover, our results show that dRYBP can bind to histones H2A and H2B as well as to the ubiquitylated variants H2Aub

and H2Bub (Figure 9D). Therefore we further investigated the role of dRYBP in epigenetic regulation mediated by PcG and trxG proteins.

2.1. The dRYBP protein biochemically interacts with SCE/dRING, dKDM2 and dBRE1

To gain insight into the function of dRYBP in epigenetic regulation we performed a mass spectometry analysis to search for dRYBP interacting proteins (Simón, R. *et al*, manuscript in preparation) (Simón, 2013) using the dRYBP antibody (Bejarano et al., 2005) and *Drosophila* embryonic nuclear protein extracts (dNE). Among many different proteins, the mass spec revealed the interaction of dRYBP with the epigenetic repressors dRING and dKDM2 and with the epigenetic activator protein dBRE1 (Table 12).

Table 12. Mass spectrometric analysis of the dRYBP, SCE/dRING, dKDM2 and dBRE1 proteins

Protein	MW (kDa)	Mascot Score	Nº of unique peptides	Sequence coverage (%)
SCE/dRING (CG5595)	48	1250	16	50.1
dKDM2 (CG11033)	151	633	10	9
dBRE1 (CG10542)	119.6	620	14	17
dRYBP (CG12190)	17	346	4	40

The SCE/dRING protein is responsible for H2A ubiquitylation (H2Aub), the histone modification responsible for gene repression, and is a member of the PcG complexes PRC1 and dRAF (Buchwald et al., 2006; Gutierrez et al., 2012; Lagarou et al., 2008; Wang et al., 2004). The dKDM2 protein is in charge of H2Aub as well as H3K36me2 demethylation and forms part of the dRAF complex together with SCE/dRING and PSC proteins (Lagarou et al., 2008). On the other hand, dBRE1 protein is responsible for H2B ubiquitylation (H2Bub), the histone modification responsible for gene activation (Henry et al., 2003; Hwang et al., 2003; Nakamura et al., 2011; Osley, 2004; Wright et al., 2011). Taking into account our previous results where dRYBP is shown to bind to H2A, H2Aub, H2B and H2Bub (Figure 9D), the interaction of SCE/dRING, dKDM2 and dBRE1 with dRYBP was further analyzed.

First, we sought to confirm the interactions shown in the mass-spec analysis by Co-immunoprecipitation (CoIP) experiments using dNE and the dRYBP antibody (Bejarano et al., 2005). The samples were resolved by SDS-PAGE and then analyzed by WB using the antibodies against SCE/dRING, dKDM2 (Lagarou et al., 2008) and dBRE1 (van der Knaap et al., 2010) proteins (Figures 10A-B). In addition, we studied the interaction with the other PcG proteins PSC, PC and PH (Lagarou et al., 2008), which did not appear in the mass spectometry

data of dRYBP-interacting proteins but have been shown to form complexes with SCE/dRING and/or dKDM2 proteins (Lagarou et al., 2008; Schuettengruber and Cavalli, 2010; Shao et al., 1999). As a negative control, detection of EZ protein was used and the reverse CoIPs were performed to validate all of the interactions (Figure 10).

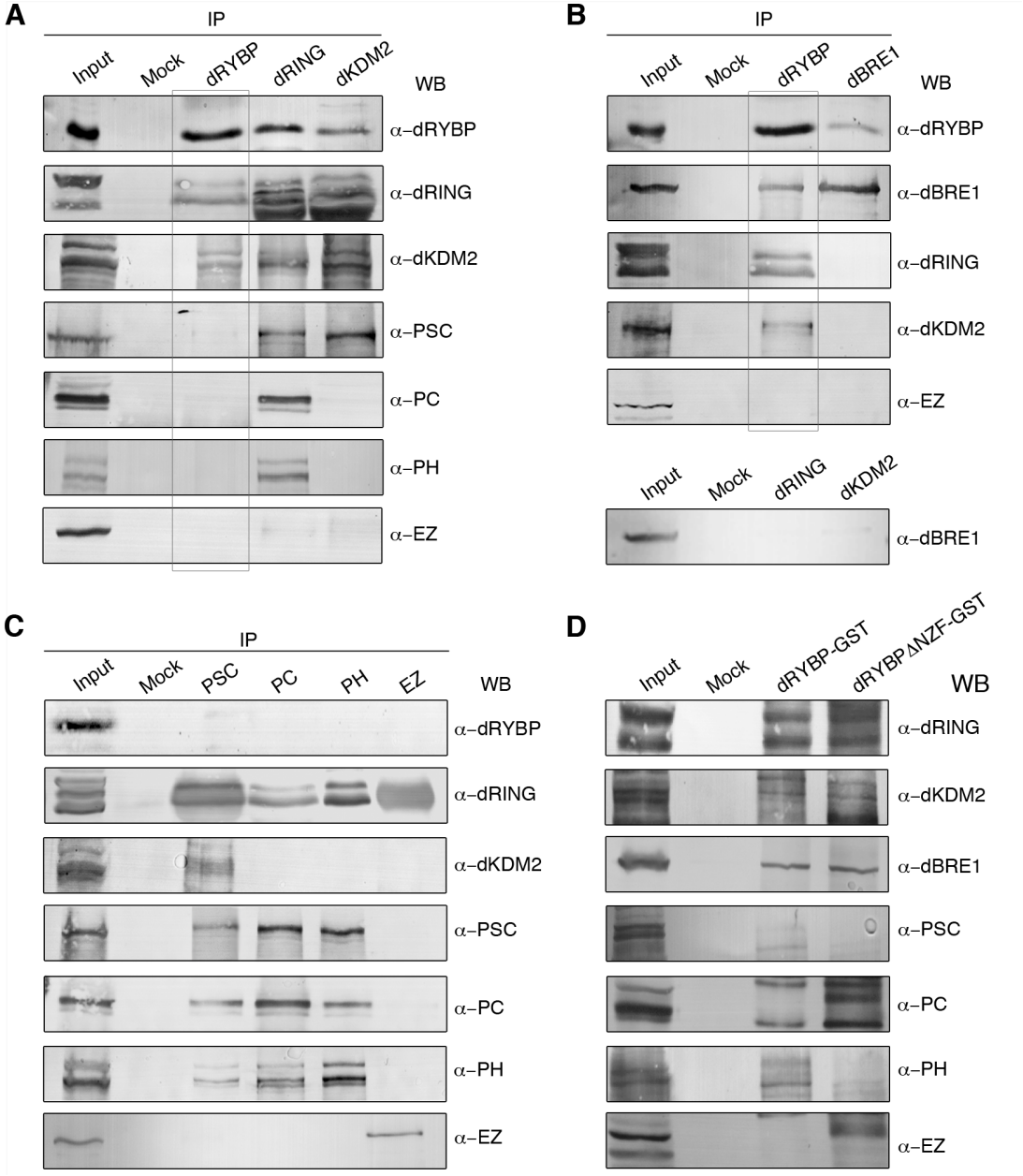


Figure 10. Biochemical interactions of dRYBP, SCE, dKDM2 and dBRE1. (**A**) dNE (Input) or pre-immune serum (Mock) were immunoprecipitated (IP) using antibodies against dRYBP, dRING or dKDM2. Eluted proteins were analyzed by WB with the corresponding antibodies. Note that dRYBP biochemically interacts with dRING and dKDM2 but not with PSC, PC, PH or EZ (rectangle). (**B**) dNE (Input) or pre-immune serum (Mock) were inmunoprecipitated (IP) using antibodies against dRYBP, dBRE1, dRING and dKDM2. Eluted proteins were analyzed by WB with the indicated antibodies. Note that dRYBP biochemically interacts with dBRE1, dRING and dKDM2 (rectangle), and that BRE1 does not interact with SCE, dKDM2 or EZ. (**C**) dNE (Input) or pre-immune serum (Mock) were immunoprecipitated (IP) with α–PSC, α–PC, α–PH and α–EZ antibodies. Eluted proteins were analyzed by WB with the indicated antibodies. Note dRYBP does not interact with the tested proteins. (**D**) GST-pulldown assays performed using dNE (Input) incubated with GST beads (Mock) or the constructs dRYBP-GST or dRYBPΔNZF-GST. Eluted proteins were analyzed by WB with the corresponding antibodies. Note that dBRE1, dRING and dKDM2 proteins interact with both dRYBP-GST and dRYBPΔNZF-GST proteins.

Figures 10A and C show that dRYBP protein interacts biochemically with SCE/dRING and dKDM2 but not with PSC, PC, PH or EZ. The results confirm the interactions previously described between dRING and dKDM2, PSC, PC and PH (Schuettengruber and Cavalli, 2010) and between dKDM2, dRING and PSC (Lagarou et al., 2008) (Figures 10A and C). On the other hand, Figure 10 shows that dRYBP interacts biochemically with dBRE1 protein and that dBRE1 does not interact with SCE/dRING, dKDM2 and EZ (Figure 10B), suggesting that dRYBP interacts molecularly with dBRE1 independently from other PcG proteins.

As previously shown in this thesis work, dRYBP binds to ubiquitylated proteins through its NZF domain (Figure 9C); therefore we studied whether this NZF domain was also involved in the binding of dRYBP to the PcG and trxG proteins. To solve this question we performed a GST-pulldown using the fusion proteins dRYBP-GST and dRYBPΔNZF-GST previously described in this work (Figure 9B). In this case we used dNE as input for the GST-pulldown to study protein interactions in the same conditions as the CoIPs (Figure 10D). The results show, in accordance with the results from the CoIPs experiments, that dRYBP interacts with SCE/dRING, dKDM2 and dBRE1 but does not associate with PSC, PC, PH and EZ (Figure 10D). Interestingly dRYBPΔNZF-GST also binds to SCE/dRING, dKDM2 and dBRE1 but not to PSC, PC, PH and EZ, suggesting that the NZF domain is not required for the interaction of dRYBP with the PcG and trxG proteins (Figure 10D).

Taken together, the above results show that dRYBP interacts biochemically with the repressor proteins dRING and dKDM2, with the activator dBRE1 and that it does no interact with any other PcG protein member studied (PSC, PC, PH and EZ). Therefore these interactions reveal that dRYBP may be defining novel protein complexes, one with dKDM2, dRING that we have denoted dRRK (*Drosophila* dRING, dRYBP and dKDM2) and a different complex with dBRE1, that we have denoted dRB (*Drosophila* dRYBP and dBRE1). Additionally, our results show that the NZF domain of dRYBP is not necessary for binding to the PcG and trxG proteins.

2.2. *dRYBP* genetically interacts with *Sce/dRing*, *dKDM2* and *dBRE1*

The molecular interactions between dRYBP and SCE/dRING and dKDM2 and dBRE1 (Figure 10), suggested a genetic interaction between these genes. We have previously shown that *dRYBP* genetically interacts with *Sce/dRing* (Gonzalez et al., 2008) therefore, we hypothesized that *dRYBP* could also be interacting genetically with *dkdm2* to control gene silencing and with *dBre1* to control gene activation.

To probe this hypothesis, we studied the genetic interactions between *dRYBP* and *Sce/dRing*, *dkdm2* and *dBre1* using the homeotic phenotypes associated with the inactivation of *PcG* and *trxG* genes (Figure 11). We analyzed the transformation of meta (L3) and mesothoracic (L2)

legs into prothoracic (L1) legs (L2-L3 to L1, Figure 11A-B) as a *PcG* mutant-associated phenotype and the transformation of the fifth (A5) abdominal segment towards the forth (A4) abdominal segment (A5 to A4, Figure 11C-D) as a *trxG* mutant-associated phenotype.

First, we analyzed the penetrance of the transformation of L2-L3 into L1 of *dRYBP* and *dkdm2* double mutant flies. Neither loss of *dRYBP* function (Gonzalez et al., 2008), loss of *dkdm2* function (Lagarou et al., 2008) nor the double mutant showed L3-L2 transformations to L1. Thus we studied the phenotypes in a sensitized mutant genetic background using *Polycomb* (*Pc*) mutant heterozygous mutant flies (Pc^3/+). 50% of the Pc^3/+ flies present L3-L2 transformation to L1 (Figure 11E). Flies $dRYBP^1/dRYBP^1$; Pc^3/+ present a similar frequency of the homeotic phenotype to Pc^3/+ flies (Figure 11E), suggesting that *dRYBP* and *Pc* do not interact genetically. Flies $dkdm2^{KG04325}/Pc^3$ show an increase in the penetrance of the L2-L3 transformations to L1 phenotype (90%, Figure 11E) when compared to Pc^3/+ flies (50% L3-L2 to L1, Figure 11E) suggesting, as previously described, that *dkdm2* is an enhancer of *Polycomb* (Lagarou et al., 2008). When *dRYBP* and *dkdm2* are concomitantly inactivated ($dRYBP^1/dRYBP^1$; $dkdm2^{KG04325}/Pc^3$) the frequency of the L3-L2 transformation to L1 is highly reduced from 90% to 26% of the flies (Figure 11E) suggesting that *dRYBP* counteracts the *dkdm2*-mediated repression.

Next, we analyzed the genetic interactions with *Sce/dRing*. Heterozygous Sce^1/+ mutant flies do not show L3-L2 transformation to L1 (Figure 11F, (Breen and Duncan, 1986; Gorfinkiel et al., 2004)) even when the *dkdm2* expression is concomitantly inactivated ($Sce^1/dkdm2^{KG04325}$, Figure 11F). However, simultaneous inactivation of *dRYBP* and *Sce/dRing* ($dRYBP^1/dRYBP^1$; Sce^1/+) produces L2-L3 transformation to L1 with a high frequency (86%, Figure 11F, (Gonzalez et al., 2008)) suggesting that *dRYBP* and *Sce/dRing* are enhancers of *Pc*. Notably, the penetrance of this phenotype is highly decreased from 86% to 2% when the levels of *dkdm2* expression are reduced ($dRYBP^1/dRYBP^1$; $Sce^1/dkdm2^{KG04325}$) (Figure 11F). Thus, these results indicate that *dkdm2* counteracts the *dRYBP/Sce*-mediated repression.

Finally, we studied the genetic interaction between *dRYBP* with *dBre1* using the *trxG* mutant-associated phenotype A5 transformation to A4 (Figure 11C-D). We have previously described $dRYBP^1$ homozygous mutant flies do not show any homeotic phenotype (Bejarano et al., 2005; Gonzalez et al., 2008). As shown in Figure 11G, $dBre1^{kim1}$ homozygous mutant flies ($dBre1^{kim1}/dBre1^{kim1}$) show with high penetrance the A5 to A4 transformation (80%, Figure 11G). However, in the absence of *dRYBP* ($dRYBP^1/dRYBP^1$; $dBre1^{kim1}/dBre1^{kim1}$) the penetrance of this phenotype is significantly decreased from 80% to 2% (Figure 11G), suggesting that *dRYBP* suppresses the activator function of *dBre1*.

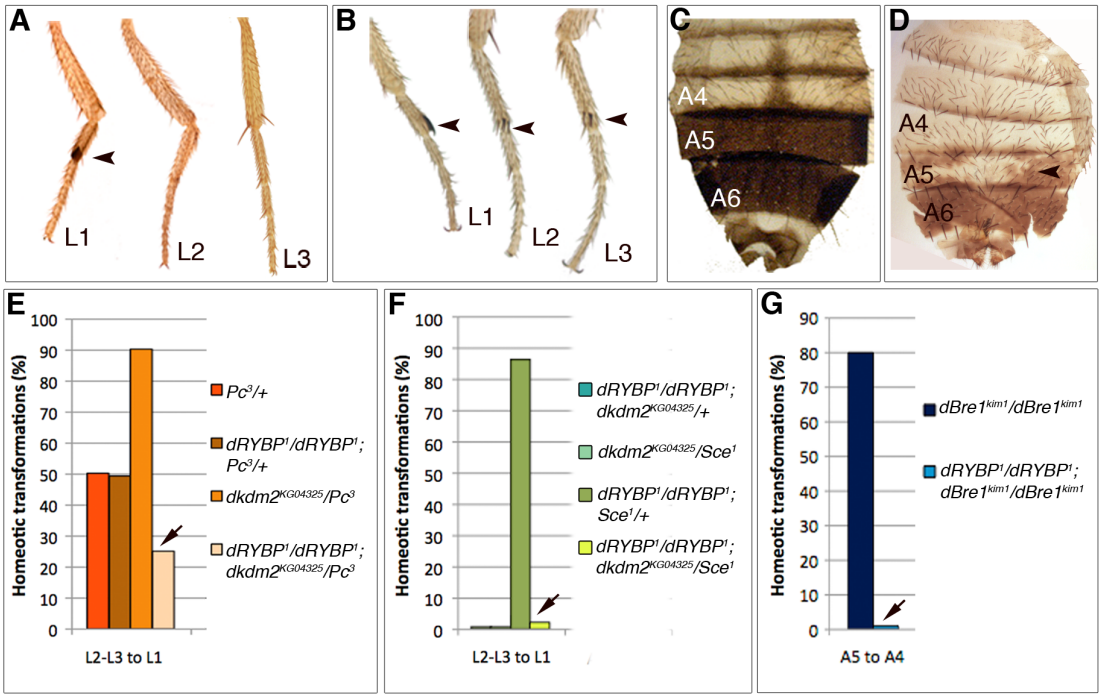


Figure 11. *dRYBP* genetically interacts with *Sce*, *dkdm2* and *dBre1*. (**A**) Wild type male legs (L1, L2 and L3). The L1 leg presents the sex comb located at the leg basitarsi (arrowhead) that is not present in L2 or L3. (**B**) *dRYBP*^{1}/*dRYBP*^{1}; *Pc*^{3}/*dkdm2*^{KG04325} male legs with L2-L3 transformation into L1 showing ectopic sex combs on L2 and L3 legs (arrowheads). (**C**) Wild type male abdomen. The A4, A5 and A6 segments are indicated. Note the pigmentation of A5 and A6. (**D**) *Bre1*^{kim1}/*Bre1*^{kim1} male abdomen showing patches of de-pigmentation (arrowhead) in the A5 and A6 segments indicative of a partial transformation to A4. (**E**) Genetic interaction between *dRYBP* and *dkdm2* in a *Pc* mutant background. Graph represents the frequency of the indicated phenotypes in flies of the indicated genotypes (n= 100 for all genotypes). Note that the frequency of the homeotic transformation in *dRYBP*^{1}/*dRYBP*^{1}; *dkdm2*^{KG04325}/*Pc*^{3} flies is reduced in comparison to *dkdm2*^{KG04325}/*Pc*^{3} flies (arrow). (**F**) Genetic interaction between *dRYBP*, *dkdm2* and *Sce*. Graph represents the frequency of the indicated phenotype in flies of the indicated genotypes (n=100 for *dRYBP*^{1}/*dRYBP*^{1}; *dkdm2*^{KG04325} /+ and *dRYBP*^{1}/*dRYBP*^{1}; *Sce*^{1}/+; n= 45 for *Sce*^{1}/*dkdm2*^{KG04325} ; n= 70 for *dRYBP*^{1}/*dRYBP*^{1}; *Sce*^{1}/*dkdm2*^{KG04325}). Note that the frequency of the homeotic transformation in *dRYBP*^{1}/*dRYBP*^{1}; *Sce*^{1}/*dkdm2*^{KG04325} flies is decreased in comparison to *dRYBP*^{1}/*dRYBP*^{1}; *Sce*^{1}/+ flies (arrow). (**G**) Genetic interaction between *dRYBP* and *dBre1*. Graph represents the percentage of the indicated phenotype in flies of the indicated genotype (n=100 in both cases). Note that the frequency of the homeotic transformation in *dRYBP*^{1}/*dRYBP*^{1}; *dBre*^{kim1}/ *dBre*^{kim} flies is reduced in comparison to *dBre*^{kim1}/*dBre*^{kim1} flies (arrow).

Taken together, the results from the study on the genetic interaction of *dRYBP*, *dkdm2* and *Sce/dRing*, indicate that dRYBP associates with these proteins to counteract and alleviate the dKDM2 and SCE mediated repression. Moreover, the results from the genetic interaction of *dRYBP* and *dBre1* indicate that dRYBP associates with dBRE1 protein to counteract and alleviate the dBRE1 mediated activation.

2.3. Biochemical function of dRYBP and dRYBP-containing complexes

PcG and trxG proteins are known to post-translationally modify histones to maintain repressed or activated transcriptional states of their target genes. Each of the proteins has a specific biochemical activity. For instance, SCE/dRING is responsible for H2Aub (Buchwald et al., 2006; Cao et al., 2005; Gutierrez et al., 2012; Lagarou et al., 2008), dKDM2 is responsible

for enhancing H2Aub and for H3K36me2 demethylation (Lagarou et al., 2008) while dBRE1 is in charge of H2Bub (Hwang et al., 2003; Nakamura et al., 2011). Due to the interaction of dRYBP with these proteins (Figure 10) the biochemical function of dRYBP was analyzed as well as its biochemical function together with its interactors.

2.3.1. dRYBP affects levels of histone post-translational modifications

To study the biochemical function of dRYBP in epigenetic regulation we checked the levels of specific histone modifications in wild type *Drosophila* S2 cells (Mock) and in S2 cells where *dRYBP* had been inactivated (dRYBP Knock Down, KD) (Figure 12).

First, we generated a dsRNA to inactivate *dRYBP* expression and subsequently analyzed the levels of dRYBP protein in dRYBP KD cells by WB in comparison to control S2 cells to confirm the efficiency of *dRYBP* inactivation (Figure 12). The results show that the levels of dRYBP protein in dRYBP KD S2 cells are almost completely absent in comparison with the levels of dRYBP in wild type cells (Mock).

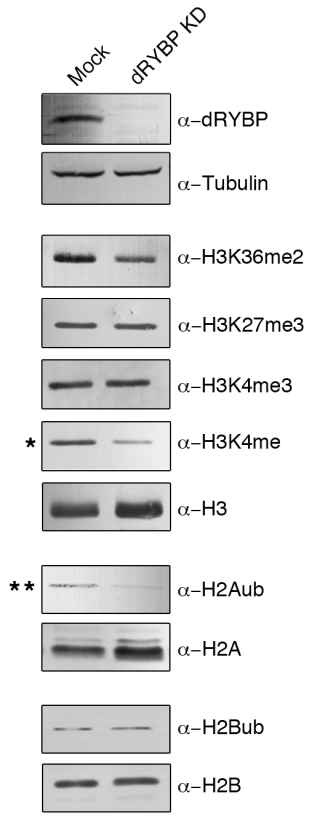


Figure 12. dRYBP affects levels of histone post-translational modifications. (**A**) S2 cells were mock-treated or treated with dsRNA against dRYBP (KD). Protein extracts were analyzed by WB using antibodies against dRYBP and Tubulin. Note dRYBP protein levels are depleted in dRYBP KD in comparison to the Mock. Histone acid extraction was performed and analyzed by WB with the corresponding antibodies to label histone marks. Note dRYBP KD decreases levels of H4K3me (*) and H2Aub (**).

Next, histones were extracted from dRYBP KD S2 cells and the levels of post-translational modifications were analyzed by WB in comparison to histones extracted from control S2 cells (Mock). The results show that in the absence of dRYBP expression there is a decrease in the ubiquitylation of histone H2A (H2Aub, Figure 12**) and a decrease in the methylation H3K4 (H3K4me, Figure 12*). However, *dRYBP* inactivation does not significantly affect the levels of H3K36 dimethylation (H3K36me2), H3K27 trimethylation (H3K27me3), H4K3 trimethylation (H4K3me3) and H2B ubiquitylation (H2Bub).

2.3.2. dRYBP modulates levels of H2Aub, H3K3me2 and H2Bub

We next studied the effect of the inactivation of *dRYBP* together with the inactivation of *Sce/dRingG*, *dkdm2* and *dBre1* on the levels of histone post-translational modifications. For that

we used dsRNAs to inactivate the expression of *dRYBP* (dRYBP KD), *Sce/dRing* (dRING KD), *dkdm2* (dKDM2 KD) (Lagarou et al., 2008) and *dBre1* (dBRE1 KD) (van der Knaap et al., 2010). The dsRNA of PC was used as a negative control to inactivate *Pc* expression (PC KD) (Lagarou et al., 2008). Double KDs were also generated using dsRNAs to concomitantly inactivate *dRYBP* and *Sce/dRing* expression (dRYBP+dRING KD), *dRYBP* and *dkdm2* expression (dRYBP+dKDM2 KD), *dRYBP* and *dBre1* expression (dRYBP+dBRE1 KD), and *dRYBP* and *Pc* expression (dRYBP+PC KD). First, protein levels of single and double KDs were analyzed by WB and compared to protein levels in wild type S2 cells (Mock). Figure 13 shows that all dsRNAs are highly effective inactivating the expression of their target. Moreover, Figure 13 shows dsRNAs only affect their targeted genes and do not produce any significant differences in the expression levels of the other genes.

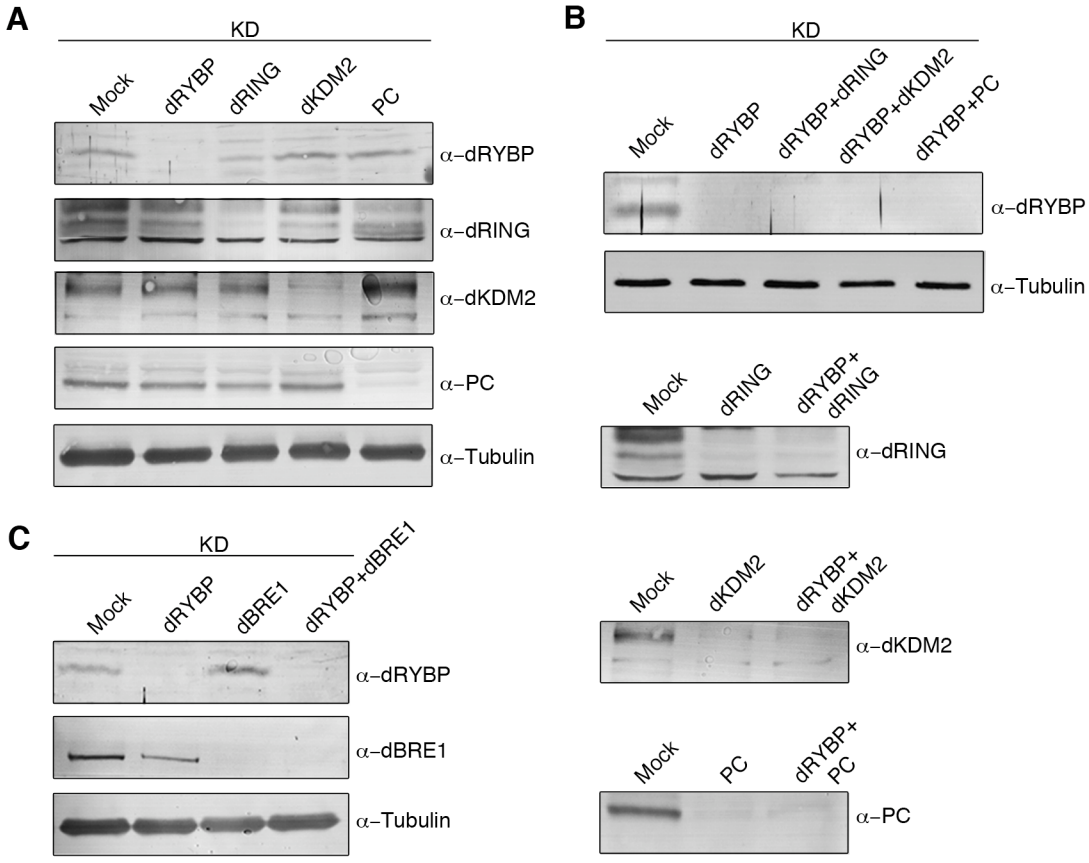


Figure 13. dsRNAs efficiently inactivate targeted gene expression. S2 cells were either mock-treated or incubated with dsRNAs against dRYBP, dRING, dKDM2 or PC for single KD (**A**), with dsRNAs against dRYBP and dRING, dKDM2 or PC for double KDs (**B**) and with dsRNAs against dRYBP or/and dBRE1 for single and double KDs (**C**). Protein extracts from S2 cells were analyzed by WB using the corresponding antibodies and α–Tubulin antibody for loading control. Note that all the tested dsRNAs inactivate gene expression of target genes and do not affect other protein levels.

Next, histone extraction from the corresponding S2 cells was performed and the levels of post-translational modifications were analyzed comparing Mock, single and double KDs. Figure

14A shows that H2Aub is slightly decreased in dRYBP KD (35%). Moreover H2Aub levels are mildly reduced in dKDM2 KD (56%), severely decreased in dRING KD (95%) and not affected in PC KD (Figure 14A) consistent with what is known from the activity of dRING, dKDM2 and PC (Cao et al., 2005; Lagarou et al., 2008). Moreover, the results also show that the decrease in the levels of H2Aub in double KDs, dRYBP+dRING KD (88%), dRYBP+dKDM2 KD (51%) and dRYBP+PC KD (46%), are similar if not identical when compared to the reduction of the levels of H2Aub in the single dRING KD (95%), dKDM2 KD (56%) and dRYBP KD (35%) respectively (Figure 14A). These results suggest that dRYBP promotes, directly or indirectly, H2A ubiquitylation together with dRING and dKDM2.

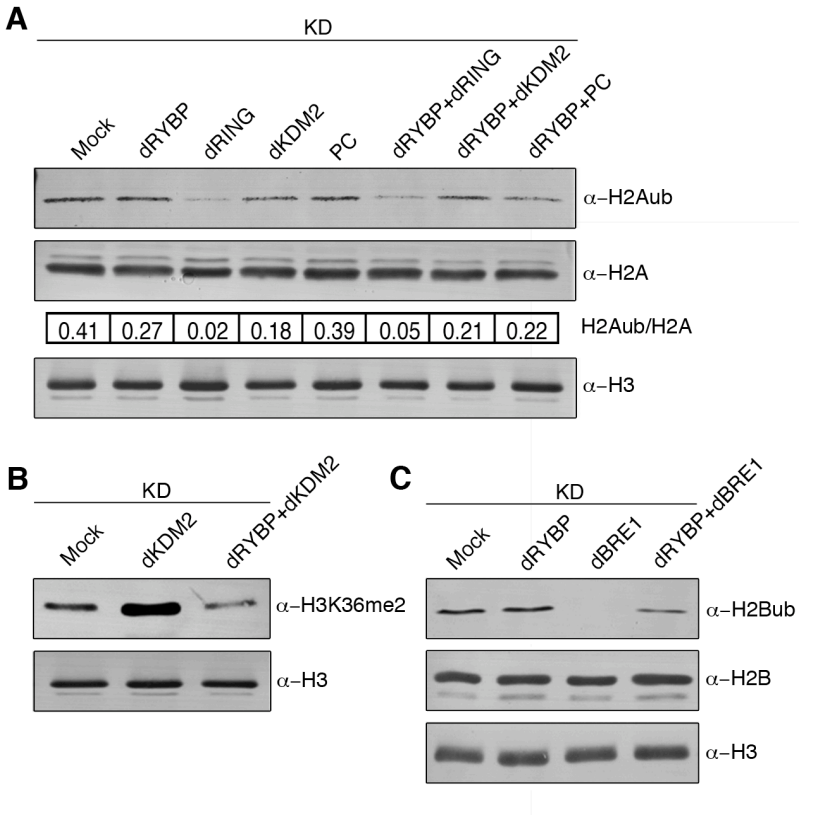


Figure 14. dRYBP modulates levels of H2Aub, H3K36me2 and H2Bub. (**A-C**) S2 cells mock-treated or incubated with the corresponding dsRNAs for single and double KDs. Histone acid extraction was prepared and WB with the corresponding antibodies. (**A**) Histones detected with α–H2Aub and with α–H2A and α–H3 as controls. The intensity of the bands corresponding to H2Aub and H2A was measured and quantified using Fiji imaging software calculating the H2Aub/H2A ratios. (**B**) Histones detected with α–H3K36me2 and α–H3 as a control. Note dRYBP+ dKDM2 KD levels of H3K36me2 decrease in comparison to dKDM2 KD. (**C**) Histones detected with α–H2Bub and with α–H2B and α–H3 as controls. Note dRYBP+dBRE1 KD levels of H2Bub increase compared to dBRE1 KD

We further analyzed the levels of H3K36me2 (Figure 14B). The inactivation of *dkdm2* in dKDM2 KD cells produces an increase in H3K36me2 levels (Figure 14, (Lagarou et al., 2008)) but dRYBP KD does not affect levels of H3K36me2 (Figures 12). However, the concomitant

inactivation of *dRYBP* and *dkdm2* (dRYBP+dKDM2 KD) produces a decrease of the H3K36me2 levels in comparison to single dKDM2 KD, which are very similar to the wild type levels of H3K36me2 (Figure 14B). These results suggest that inactivation of *dRYBP* together with *dkdm2* counteracts the high levels of H3K36me2 induced by inactivation of *dkdm2*, suggesting that in a wild type situation dRYBP counteracts the demethylation induced by dKDM2 in the H3K36me2.

Finally we analyzed the effect on H2Bub. The inactivation of *dBre1* induces a complete depletion of H2Bub (Figure 14C, (Hwang et al., 2003; Walter et al., 2010)) and loss of function of *dRYBP* does not affect H2Bub (Figures 12 and 14C). However, the simultaneous inactivation of *dRYBP* and *dBre1* expression (dRYBP+dBRE1 KD) partially restores the levels of H2Bub (Figure 14C). These results indicate that inactivation of *dRYBP* together with *dBRE1* counteracts the reduction of H2Bub induced by inactivation of *dBRE1*, suggesting that in a wild type situation dRYBP suppresses the ubiquitylation of H2B mediated by dBRE1.

In summary, results from these experiments show that dRYBP interacts with SCE/dRING, dKDM2 and dBRE1 proteins but not with the PcG proteins PSC, PC, PH and EZ (Figure 10). Moreover, the NZF domain of dRYBP is not necessary for the interaction with these proteins (Figure 10D), suggesting that another dRYBP-domain may be required for it to bind to SCE/dRING, dKDM2 and dBRE1. In addition, our results show that dRYBP is able to modulate the levels of H2Aub, H3K36me2 and H2Bub (Figure 14), indicating its role in epigenetic regulation of gene expression mediated by PcG and trxG. Moreover, new evidence is emerging showing chromatin-independent functions of the PcG and trxG proteins (Lecona et al., 2013; Mohd-Sarip et al., 2012; Wen et al., 2014), suggesting that these proteins are involved in other functions in addition to the regulation of gene expression through histone modifications.

3. FUNCTIONAL STUDY OF dRYBP IN APOPTOSIS REGULATION

Several experimental observations implicate dRYBP protein in the regulation of apoptosis pathways. First, lack of *dRYBP* function produces a variety of phenotypes, including organ size reduction (Gonzalez et al., 2008). Second, high levels of dRYBP protein induce apoptosis (Gonzalez and Busturia, 2009), with its human counterpart RYBP/DEDAF doing so exclusively in transformed cells (Novak and Phillips, 2008). Third, both the fly and the vertebrate RYBP interact with a variety of apoptosis-related proteins (Chen et al., 2009; Gonzalez and Busturia, 2009; Novak and Phillips, 2008; Stanton et al., 2007), however the molecular mechanisms controlling dRYBP/RYBP-mediated apoptosis are poorly understood.

3.1. *dRYBP*-interacting proteins include the SCF E3 ubiquitin ligase complex components

To begin to decipher the function of dRYBP in the regulation of the mechanisms of cell death, we identified dRYBP-interacting proteins within the list of dRYBP-associated proteins resulting from the mass spectrometry experiments (Simón, R. *et al*, manuscript in preparation) (Simón, 2013). We found that dRYBP associates with members of the SCF E3 ubiquitin ligase complex (Table 13), previously shown to regulate the apoptotic pathway in mammals by protein ubiquitylation (Tan et al., 2006). Taking into account that the dRYBP protein contains a UBD (Figure 7) (Alam et al., 2004; Bejarano et al., 2005), we hypothesized that dRYBP might be regulating apoptosis together with the SCF complex. Thus, we sought to investigate the interaction with the SCF complex members.

Table 13. Mass spectrometric analysis of the dRYBP, SKPA, dCUL1 and Pall proteins

Protein	MW (kDa)	Mascot Score	Nº of unique peptides	Sequence coverage (%)
dRYBP (CG12190)	17	346	4	40
SKPA (CG16983)	19	320	5	47.5
dCUL1 (CG1877)	89.5	92	2	3.5
Pall (CG3428)	36	118	2	6.5

The SCF complex is composed by the scaffold proteins SKPA and dCUL1 and the F-box (Bocca et al., 2001; Jackson and Eldridge, 2002), which is responsible for substrate specificity (Skowyra et al., 1997) and ultimately, for different biological functions (Cardozo and Pagano, 2004; Heriche et al., 2003; Inuzuka et al., 2011; Silverman et al., 2012). The mass spec data revealed that dRYBP interacts with the SCF complex proteins SKPA and dCUL1 (Table 13). The only F-box in the dRYBP mass-spec data was Pallbearer (Pall), previously shown to interact with SKPA and dCUL1 in the SCF complex to promote engulfment of cells (Ferrandon, 2007). We analyzed the inactivation of *Pall* and found that it does not produce any visible phenotype in the wing, nor did we find that inactivation of *Pall* induced apoptosis in the wing imaginal discs (Appendix 2). Therefore, we did not study the interaction of *Pall* and *dRYBP* and their role in the regulation of apoptosis any further.

Searching for other F-boxes that could form part of this complex, we found that the protein Slimb (Slmb) interacts with both SKPA and dCUL1 (Bocca et al., 2001). Moreover, mutant cell clones of *slmb* induce apoptosis (Miletich and Limbourg-Bouchon, 2000). Although Slmb does not appear in the dRYBP-mass-spec data, we selected this protein to study the interaction with dRYBP and the SCF complex in the regulation of apoptosis.

3.2. Subunits of the SCF E3 ligase complex interact with dRYBP

First, we analyzed the biochemical interactions between dRYBP and SKPA and dCUL1 by Co-immunoprecipitation of dNE using an anti-dRYBP antibody (Bejarano et al., 2005), followed by identification of the associated proteins by WB with antibodies against SKPA(Hughes et al., 2008) and dCUL1 (Table 7). Figure 15 shows that SKPA and dCUL1 co-immunoprecipitate with dRYBP. We could not test the biochemical interaction of the F-box Slmb with dRYBP because there was not an anti-Slmb antibody available at the time when we performed the experiments.

These results demonstrate the biochemical interaction between dRYBP and SKPA and dCUL1 and suggest that dRYBP participates, together with the SCF complex, in the process of protein ubiquitylation.

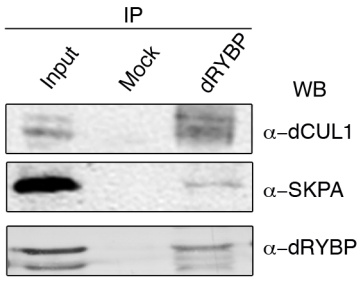


Figure 15. dRYBP biochemically interacts with SKPA and dCUL1. (**A**) dNE (Input) were IP using Sepharose A beads only (Mock) or Sepharose A beads coupled to α-dRYBP antibody. The precipitates were analyzed by WB using antibodies against dCUL1, SKPA or dRYBP proteins. The SKPA Input sample and the SKPA IP sample were not diluted. The dCUL1 and dRYBP Input samples were 50% diluted while the dCUL1 IP and dRYBP IP samples were not diluted.

3.3. *dRYBP* genetically interacts with the SCF complex members *skpA*, *dCul1* and *slmb*

Next, the functionality of the interactions between dRYBP and the SCF complex was studied by correlating the phenotypes associated with the lack of *dRYBP* function with the phenotypes associated with loss of the *SCF* complex function. Among other phenotypes, *dRYBP* homozygous mutants and inactivation of *dRYBP* in flies show a reduction of the wing size (Figure 16B, (Gonzalez et al., 2008)). Using this wing phenotype as readout, we analyzed the genetic interactions between *dRYBP* and the SCF complex components *skpA*, *dCul1* and *slmb*. Because loss of function mutations in the *dCul1*, *slmb* and *skpA* genes are embryonic lethal (Jiang and Struhl, 1998; Murphy, 2003; Spradling et al., 1999), the GAL/UAS system (Brand et al., 1994) was used to inactivate their function in the wing imaginal disc.

Flies *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* show wings with a substantial size reduction (Figure 16C) due to a decrease in the levels of SKPA protein (see Figure 17 for the efficiency of the *UAS-skpA*$_{RNAi}$ and *UAS-skpA*^{OB} transgenic lines, for the cellular localization of SKPA, the specificity of the anti-SKPA antibody and for additional larval phenotypes associated with loss of *skpA* function). Moreover, this small wing phenotype (Figure 16C) is not due to de-

repression of the homeotic protein Ubx in the wing imaginal discs of *sdGal4>UAS-skpA*$_{RNAi}$ larvae (Appendix 3).

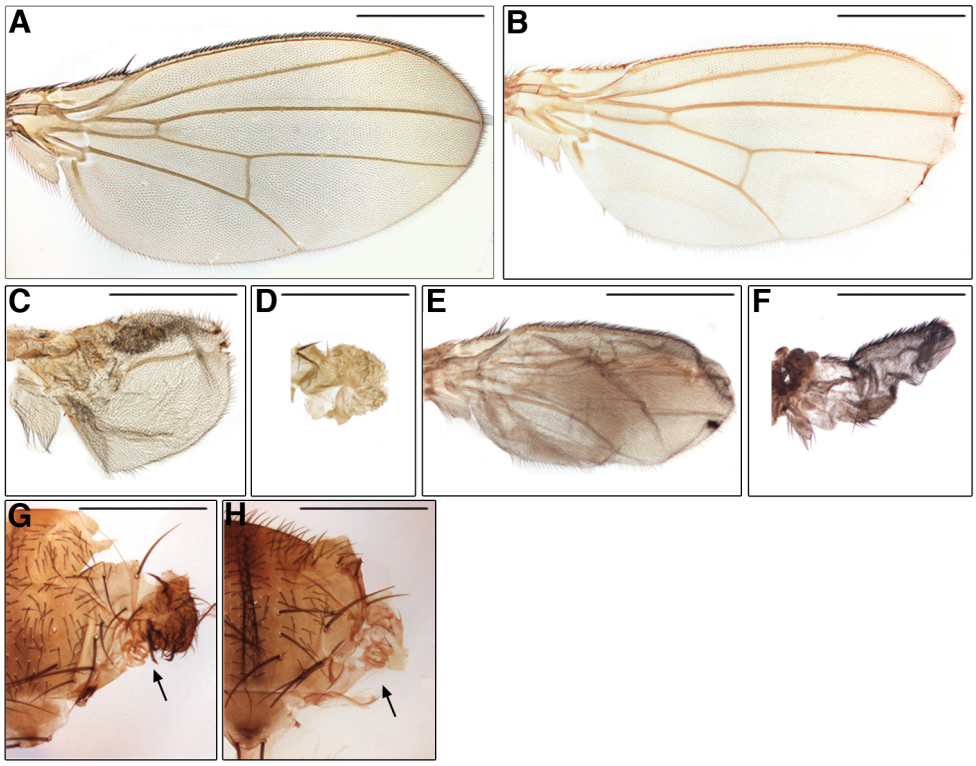


Figure 16. *dRYBP* genetically interacts with *SCF* complex members. (**A-H**) *dRYBP* and *SCF* complex genetic interactions. (**A**) Wing from control *sdGal4* fly. (**B**) Wing from *sdGal4>UAS-dRYBP*$_{RNAi}$*/UAS-GFP* fly. Note the moderate wing size reduction compared to control (A). (**C**) Wing from *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* fly. (**D**) Wing from *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ fly. Note the increase in wing size reduction due to the simultaneous inactivation of *skpA* and *dRYBP*. (**E**) Wing from *sdGal4>UAS-slmb*$_{RNAi}$*/UAS-GFP* fly. (**F**) Wing from *sdGal4>UAS-slmb*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ fly. Note the increase in wing size reduction due to the simultaneous inactivation of *slmb* and *dRYBP*. (**G**) Heminotum of *sdGal4>UAS-dCul1*$_{RNAi}$*/UAS-GFP* fly. Arrow indicates the wing tissue transformed into notum. (**H**) Heminotum of *sdGal4>UAS-dCul1*$_{RNAi}$ */UAS-dRYBP*$_{RNAi}$ fly. Arrow indicates the site of wing tissue in normal flies, completely lacking in this genetic background. Scale bars represent 0.5mm

Furthermore, simultaneous inactivation of both *skpA* and *dRYBP* in *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ larvae results in a much greater wing size reduction (Figure 16D) in comparison to the wing size reduction due to the individual inactivation of either gene (Figures 16B and C), indicating the genetic interaction between *skpA* and *dRYBP*. Additionally, wings from flies *sdGal4>UAS-slmb*$_{RNAi}$*/UAS-GFP* are not properly formed (Figure 16E) and show a size reduction which is much more severe in *sdGal4>UAS-slmb*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ (Figure 16F). Finally, inactivation of *dCul1* in the wing imaginal disc of *sdGal4>UAS-dCul1*$_{RNAi}$*/UAS-GFP* larvae results in flies with rudimentary wings showing morphological features of notum (Figure 16G). However in flies *sdGal4>UAS-dCul1*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ the transformation towards notum is not even present, indicating a synergistic effect between *dRYBP* and *dCul1* (Figure 16H).

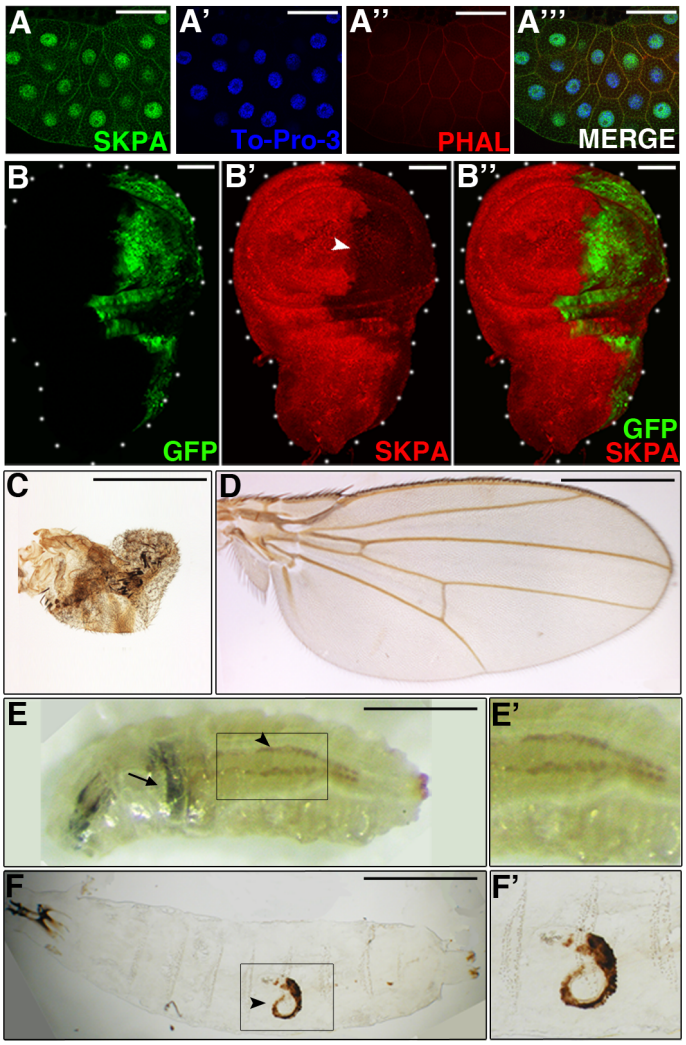


Figure 17. Analysis of SKPA expression and *skpA* mutant-associated phenotypes. (**A-A'''**) Control $y^1,Df(1)w^{67c23}$ salivary gland showing SKPA (green) (A), To-Pro-3 (blue) (A') and Phalloidin (red) expression (A''). (**A'''**) Merged images (A), (A') and (A''). (**B-B''**) *UAS-skpA*$_{RNAi}$ decreases levels of SKPA protein. Wing disc *enGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing GFP (green) (**B**) expression, labeling the *enGal4* domain in the posterior compartment and SKPA (red) (**B'**) expression. Note the decrease in SKPA expression (arrowhead) due to the *skpA* inactivation in the posterior compartment. (**B''**) Merged images (B) and (B'). Scale bars represent 100µm. (**C-D**) High levels of SKPA rescues the wing phenotype associated with *skpA* inactivation. (**C**) Wing *sdGal4>UAS-skpA*$_{RNAi}$.(**D**) Wing *sdGal>UAS-skpA*$_{RNAi}$*/UAS-skpA*^{OB}. Note the complete rescue of the wing phenotype. (**E-F**) Inactivation of *skpA* induces larval melanotic cells. (**E**) Larva *enGal4>UAS-skpA*$_{RNAi}$ showing melanotic cells in the heart (arrowhead) and salivary glands (arrow). (**E'**) Enlargement of heart melanotic cells shown in (E). (**F**) Larva *enGal4>UAS-skpA*$_{RNAi}$ showing melanotic cells in the gut (arrowhead). (**F'**) Enlargement of melanotic gut cells shown in (F). Scale bars represent 0.5 mm.

Additionally, we analyzed the genetic interactions between *dRYBP* and the components of the SCF complex studying fly survival (Figure 18). The survival of flies where *dRYBP* has been simultaneously inactivated with *skpA*, *dCul1* or *slmb* is significantly decreased in comparison to flies where *skpA*, *dCul1*, *slmb* or *dRYBP* have been separately inactivated (Figure 18).

Hence, the results indicate that *dRYBP* genetically interacts with the SCF components *skpA*, *dCul1* and *slmb*.

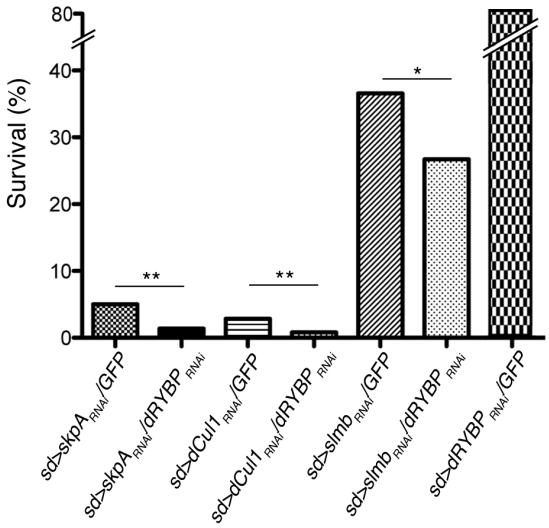


Figure 18. Function of dRYBP and the SCF complex in fly survival. Survival (percentage) of flies of the indicated genotypes. Error bars represent SEM (standard error of the mean) and asterisks denote the following p values: * p<0.05 and ** p<0.005.

3.4. Inactivation of *dRYBP* and inactivation of the *SCF* complex components induces apoptosis

We next investigated whether miss-regulation of apoptosis was responsible for the wing phenotype associated to the functional inactivation of *dRYBP*, *skpA*, *dCul1* and *slmb*. First, we analyzed the expression of activated-Caspase 3 (C3) in the wing imaginal discs of *sdGal4>UAS-dRYBP*$_{RNAi}$ larvae (Figure 19A). The results show that C3 is expressed in a small number of cells in *sdGal4>UAS-dRYBP*$_{RNAi}$ wing discs (Figure 19A) in comparison to C3 expression in wild type *sdGal4* control discs (Figure 19B). This indicates that the inactivation of *dRYBP* weakly induces apoptosis in the wing imaginal disc but significantly ($p<0.05$) increases by 35% the amount of cells undergoing apoptosis by in comparison to the number of cells undergoing apoptosis in a wild type wing disc (Figure 19D).

Subsequently, apoptosis was analyzed in wing discs of *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* larvae and we found C3 to be expressed in a large number of cells (Figure 19E) in comparison to a wild type disc (Figure 19B). Additionally, high levels of C3 protein were observed when we inactivated *skpA* using other drivers, such as *apGal4*, and in other imaginal discs, such as the leg imaginal disc, which resulted in an apoptotic adult leg phenotype (Figure 20) (to observe the inhibition of C3 expression in the presence of high levels of the baculovirus anti-apoptotic p35 protein (Hay et al., 1994) confirming the activation of the apoptotic pathway see Appendix 4).

Furthermore, we found high levels of C3 expression in *sdGal4>UAS-dCul1*$_{RNAi}$*/UAS-GFP* (Figure 19G) and *sdGal4>UAS-slmb*$_{RNAi}$*/UAS-GFP* wing imaginal discs (Figure 19I), which is consistent with previous reports that indicated that reduced *dCul1* function and mutant cell clones for *slmb* produce apoptosis(Heriche et al., 2003; Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000).

Taken together, the results indicate that the inactivation of *dRYBP*, *skpA*, *dCul1* or *slmb* induces apoptosis in the imaginal cells and therefore, may interact to inhibit apoptosis.

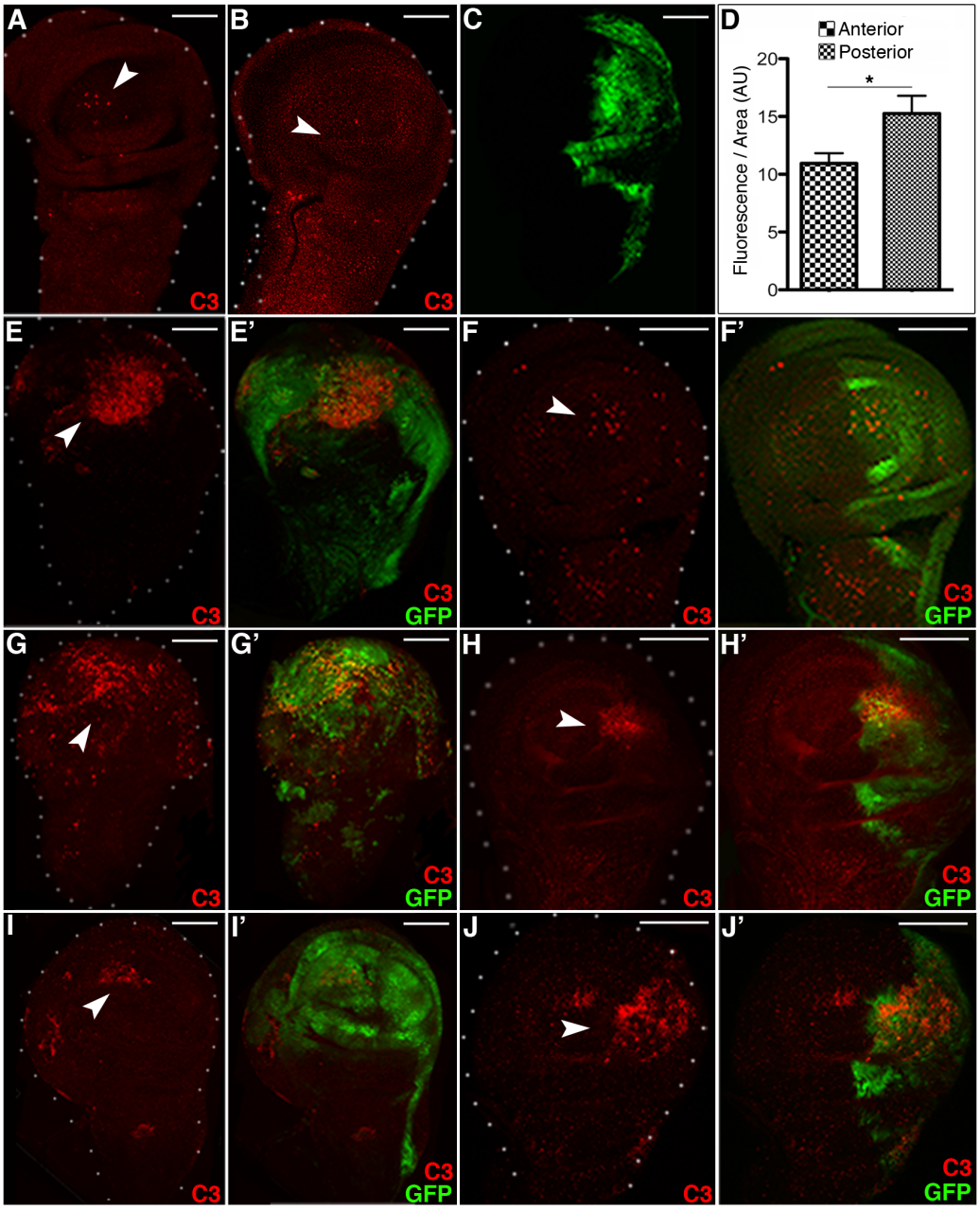


Figure 19. Inactivation of *dRYBP* and the *SCF* complex acts synergistically to induce apoptosis in wing imaginal discs. arrowheads point to apoptotic cells labeled with α-C3 (red). (**A**) Wing disc *sdGal4>UAS-dRYBP$_{RNAi}$* showing C3 expression. (**B**) Control *y^{1}, Df(1)w^{67c23}* wing disc showing C3 expression. (**C**) Wing disc *enGal4>UAS-GFP* showing the expression of GFP (green), marking of the *enGal4* domain in the posterior compartment. (**D**) Quantification of C3 expression (fluorescence/area) in the anterior and posterior compartments of *enGal4>UAS-dRYBP$_{RNAi}$/UAS-GFP* wing discs (n=21). Error bars represent SEM and asterisk denotes the following p value: * p<0.05. AU= Arbitrary Units. (**E**) Wing disc *sdGal4>UAS-skpA$_{RNAi}$/UAS-GFP* showing C3 expression. (**E'**) Merge of (E) with GFP (green) expression. (**F**) Wing disc *enGal4>UAS-dRYBP$_{RNAi}$/UAS-GFP* showing C3 expression. (**F'**) Merge of (F) with GFP (green) expression. (**G**) Wing disc *sdGal4>UAS-dCul1$_{RNAi}$/UAS-GFP* showing C3 expression. (**G'**) Merge of (G) with GFP (green) expression. (**H**) Wing disc *enGal4>UAS-skpA$_{RNAi}$/UAS-GFP* showing C3 expression. (**H'**) Merge of (H) with GFP (green) expression. (**I**) Wing disc *sdGal4>UAS-slmb$_{RNAi}$/UAS-GFP* showing C3 expression. (**I'**) Merge of (I) with GFP (green) expression. (**J**) Wing disc *enGal4>UAS-GFP;UAS-dRYBP$_{RNAi}$/UAS-skpA$_{RNAi}$*. Note that the number of cells showing C3 expression increases in response to the simultaneous inactivation of *dRYBP* and *skpA* (compare (F) and (H)). (**J'**) Merge of (J) with GFP (green) expression. Scale bars represent 100μm.

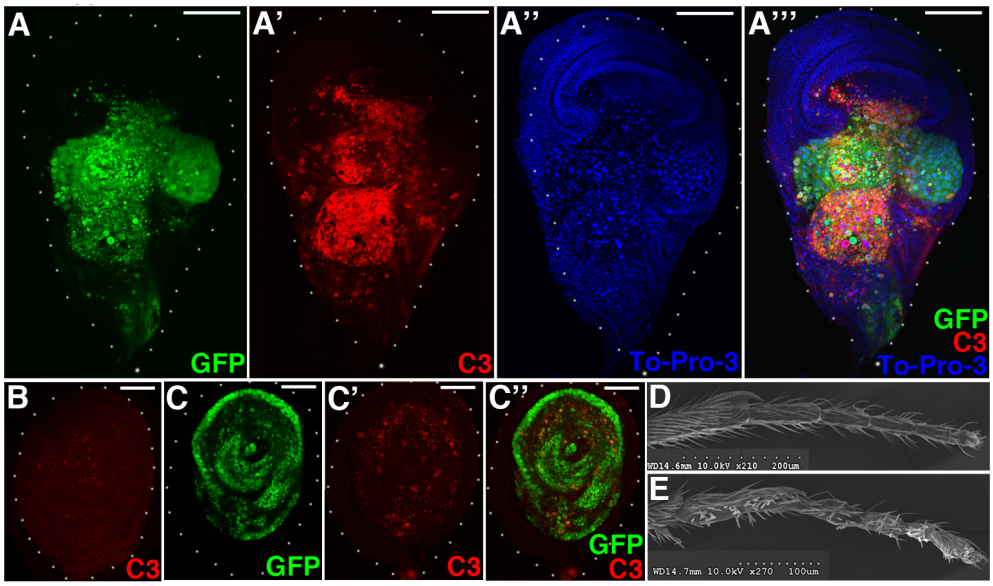


Figure 20. Inactivation of *skpA* with numerous drivers induces C3 expression and apoptotic phenotypes. (**A**) Wing disc *apGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing GFP (green) expression, labeling the *apGal4* domain in the dorsal compartment (**A'**) Wing disc *apGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing C3 (red) expression. Note the high amount of cells expressing C3 due to inactivation of *skpA*. (**A''**) Merge of images (A) and (A'). Scale bars represent 100µm. (**B**) Metathoracic leg disc y^{1},*Df(1)*w^{67c23} showing wild type C3 (red) expression. (**C**) Metathoracic leg disc *dllGal4>UAS-skpA*$_{RNAi}$ showing GFP (green) expression, labeling the *dllGal4* expression domain. (**C'**) Metathoracic leg disc *dllGal4>UAS-skpA*$_{RNAi}$ showing C3 (red) expression. Note the increase of cells expressing C3 in comparison to (B). (**C''**) Merge of images (C) and (C'). (**D**) Prothoracic leg disc *dllGal4>UAS-GFP*. Scale bar represents 200µm. (**E**) Prothoracic leg disc *dllGal4>UAS-skpA*$_{RNAi}$. Scale bar represents 100µm.

We then asked whether the apoptosis observed in the absence of *skpA* was modulated when *dRYBP* function was concomitantly inactivated. We analyzed the expression of C3 in the posterior compartment of wing imaginal discs where the function of *skpA* and *dRYBP* was simultaneously inactivated using the *enGal4* line (Figure 19C). The results show that C3 expression in *enGal4>UAS-GFP;UAS-skpA*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ (Figure 19J) is increased when compared to the C3 expression in either *enGal4>UAS-dRYBP*$_{RNAi}$*/UAS-GFP* (Figure 19F) or in *enGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* (Figure 19H) wing imaginal discs. The increase in C3 expression when *skpA* and *dRYBP* are simultaneously inactivated is consistent with the synergistic effect on the wing size reduction observed in *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-dRYBP*$_{RNAi}$ (Figure 19D) and suggests that dRYBP and the SCF complex function together to inhibit apoptosis in *Drosophila*.

3.5. The dRYBP-SCF complex regulates the intrinsic apoptotic pathway

The results show that the inactivation of the dRYBP-SCF complex induces apoptosis (Figure 19) and therefore this complex might be involved in the inhibition of cell death. To begin to decipher the molecular mechanisms by which dRYBP-SCF inhibits cell death, we asked

whether the apoptosis induced by loss of *skpA* and *dRYBP* function was dependent on the levels of expression of the components of the intrinsic apoptotic pathway; the pro-apoptotic protein Reaper (Rpr) (Yoo et al., 2002) and the anti-apoptotic protein DIAP1 (Hay et al., 1995) (Figure 8) (to observe the activation of other pathways studied such as Dmp53 (Dichtel-Danjoy et al., 2013) and the JNK pathway (Davis, 2000; Moreno et al., 2002) in the apoptosis induced by inactivation of *skpA* see Appendix 5).

3.5.1. Inactivation of *skpA* induces *rpr* transcription and increases Rpr protein levels

We used *reaper-lacZ* (*rpr-lacZ*) as a reporter of Rpr expression (Jiang et al., 2000). We examined β–GAL expression in wing imaginal discs of *sdGal4>rpr-lacZ;UAS-skpA*$_{RNAi}$ larvae. Figure 21B shows that β-GAL expression is significantly up-regulated in comparison to β-GAL expression in a wild type *rpr-lacZ* wing disc (Figure 21A) by 60%, ($p<0.005$) (Figure 21C). This result indicates that SKPA directly or indirectly regulates *rpr* transcription.

Furthermore, we sought to analyze the levels of expression of pro-apoptotic genes (Yoo et al., 2002) and their protein levels in S2 cells where *skpA* was inactivated. For that, we generated a specific dsRNA to inactivate *skpA* (skpA KD, Figures 21D and E) and measured the mRNA levels of pro-apoptotic genes *rpr* and *hid* by RT-qPCR (Figure 21D). The results show that inactivation of *skpA* in S2 cells does not affect the transcription of *hid*, but causes a significant increase in the levels of *rpr* mRNA (Figure 21D). The up-regulation of *rpr* transcription in S2 cells is in accordance with the results observed in wing imaginal discs, where *rpr-lacZ* transcription is also up-regulated (Figure 21B) (see section 3.5.6. for explanation for *dRYBP* inactivation in S2 cells).

Additionally, we studied the effect of inactivation of *skpA* on the levels of Rpr protein. For that, wild type S2 cells protein extracts were resolved by SDS-PAGE and analyzed by WB with anti-Rpr antibody (a gift from H. Steller, unpublished) and compared to S2 cells extracts were *skpA* was inactivated (skpA KD). Figure 21E shows a small but noticeable increase of Rpr expression in comparison to the undetectable Rpr expression in wild type S2 cells (Mock) (Figure 21E).

Taken together these results suggest that the apoptosis induced by *skpA* inactivation induces high levels of *rpr* mRNA and of Rpr protein.

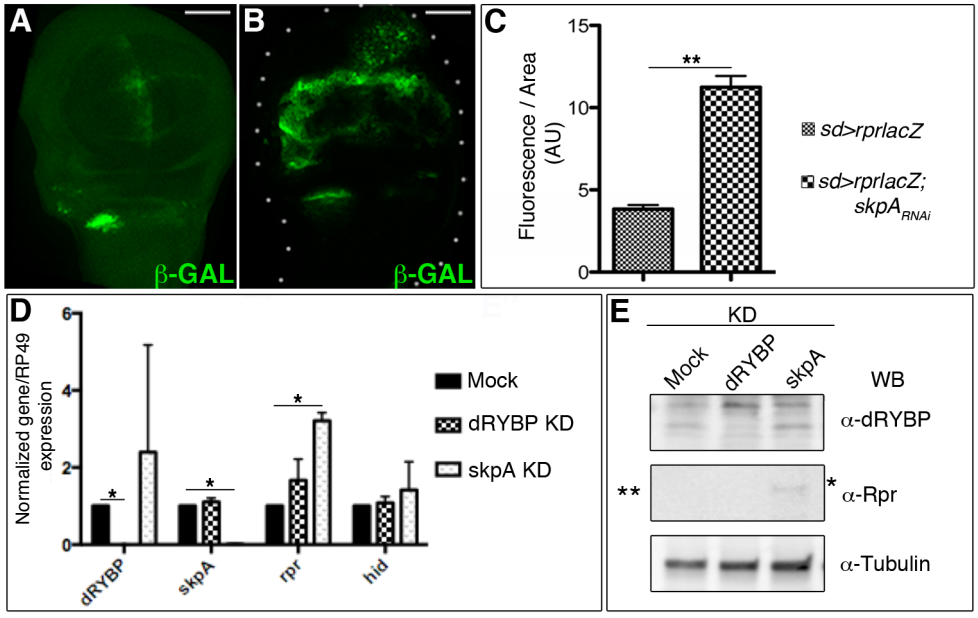


Figure 21. Inactivation of *skpA* induces *rpr* transcription and increases Rpr protein levels. (**A**) Wing disc *reaper-lacZ* (*rpr-lacZ*) showing β-GAL (green) expression. (**B**) Wing disc *sdGal4>rpr-lacZ;UAS-skpA*$_{RNAi}$ showing β-GAL (green) expression. Scale bars represent 100μm. (**C**) Quantification of β-GAL expression (fluorescence/area) in the wing imaginal discs of the indicated genotypes (n=20). Error bars represent SEM and asterisks the following p values: ** p<0.005. AU= Arbitrary Units. (**D**) mRNA levels of *dRYBP*, *skpA*, *rpr* and *hid* measured by RT-qPCR in untreated S2 cells (Mock) or treated with dsRNA against dRYBP (dRYBP KD) or skpA (SKPA KD). Error bars represent SEM of 3 different biological samples and asterisks the following p values: * p<0.01 (**E**) Protein extracts from S2 cells untreated (Mock) or treated with dsRNA against dRYBP (KD) or skpA (KD) were resolved by SDS-PAGE and immunoblotted with α-dRYBP and α-Rpr antibodies and α-Tubulin antibody as a loading control. Although Rpr protein levels are weakly expressed in skpA KD cells (*), they are significantly increased in comparison to Rpr protein levels in the Mock (**) where Rpr protein is undetectable.

3.5.2. Inactivation of *skpA*-induced apoptosis is dependent on *rpr* expression

Next we studied the effect of the inactivation of *rpr* function in the apoptosis induced by *skpA* inactivation. For that we analyzed C3 expression in wing discs where *skpA* was inactivated (*sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP*) and compared it to C3 expression in wing discs where *skpA* and *rpr* were simulateneously inactivated (*sdGal4>UAS-skpA*$_{RNAi}$*/UAS-rpr*$_{RNAi}$) (Figure 22). To control the effect of dilution of the Gal4 driver and the intrinsic variability of C3 staining, imaginal discs from *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-rpr*$_{RNAi}$ and *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* larvae were stained in the same tube. Figure 22B shows that the number of cells expressing C3 is strongly reduced when *rpr* and *skpA* are simultaneously inactivated compared to the number of cells expressing C3 when only *skpA* is inactivated (compare Figures 22A and B). Additionally, C3 expression was quantified in the area delimited by the domain of *sdGal4* in the wing pouch of *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-rpr*$_{RNAi}$ wing discs compared to *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* (Figure 22C). The results from quantifying C3 expression show a significant 75% reduction (p<0.0001) of C3 expression when *rpr* and *skpA* are simultaneously inactivated

(Figure 22C) indicating that the apoptosis induced by the inactivation *skpA* is dependent on *rpr* expression.

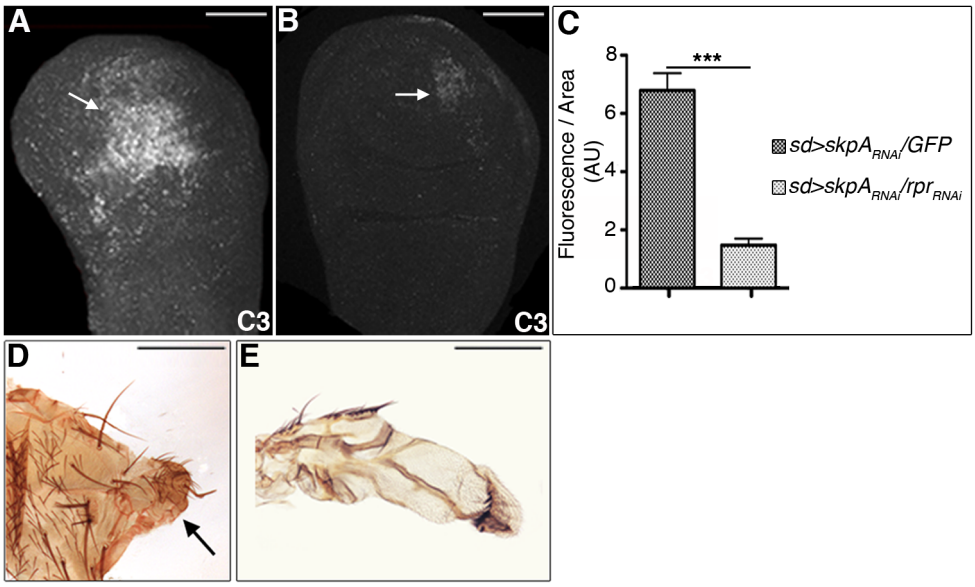


Figure 22. Apoptosis induced by inactivation of *skpA* is dependent on levels of Rpr. (**A**) Wing disc *sdGal4>UAS-skpA$_{RNAi}$/UAS-GFP* showing activated-Caspase 3 (C3) (white) expression. Arrow indicates apoptotic cells. (**B**) Wing disc *sdGal4>UAS-skpA$_{RNAi}$/UAS-rpr$_{RNAi}$* showing C3 (white) expression. Note that the number of apoptotic cells decreases in comparison to (A) (arrow). Scale bars represent 100µm. (**C**) Quantification of C3 expression (fluorescence per area) of the corresponding genotypes (n=20). Error bars represent SEM and asterisks denote the following p values: *** p<0.0001. AU= Arbitrary Units. (**D**) Heminotum of *sdGal4>UAS-rpr-HA;UAS-GFP* showing the lack of wing blade (arrow). (**E**) Wing form *sdGal4>UAS-rpr-HA;UAS-skpA^{OB}* flies . Note the rescue of the wing phenotype compare to (D). Scale bars represent 0.5mm.

3.5.3. Overexpression of SKPA rescues the phenotype induced by overexpression of Rpr protein

As shown above, *skpA* induced-apoptosis promotes *rpr* expression (Figure 21) and that this apoptosis is rescued when *rpr* is simultaneously inactivated (Figures 22B and C). To further characterize the functionality of the interaction between *rpr* and *skpA*, we studied whether overexpression of SKPA could modulate the phenotype induced by Rpr overexpression. We used different *UAS-rpr* transgenic fly lines, which were lethal, and selected *UAS-rpr-HA* (Sandu et al., 2010) because its phenotype could be modulated. Figure 22D shows that overexpression of Rpr in *sdGal4>UAS-rpr-HA/UAS-GF*P flies promotes the complete disappearance of wings, probably due to the induction of cell death mediated by high levels of Rpr protein (Ryoo et al., 2002; Yoo et al., 2002). However when SKPA and Rpr are simultaneously overexpressed in *sdGal4>UAS-rpr-HA;UAS-skpA^{OB}* flies, the wing phenotype is significantly rescued (Figure 22E). This result indicates that high levels of SKPA inhibits the apoptosis induced by Rpr overexpression and also suggests that SKPA functions to inhibit apoptosis by negatively regulating Rpr protein levels.

3.5.4. Inactivation of *skpA* induces *diap1* transcription and decreases DIAP1 protein levels

To determine if the anti-apoptotic protein DIAP1 was also involved in the apoptosis inhibition mediated by the dRYBP-SCF, we used the transgenic flies containing *diap1-lacZ* as a reporter for *diap1* expression (Hay et al., 1995). Figure 23B shows that the expression of β-GAL in *sdGal4>diap1-lacZ/UAS-skpA*$_{RNAi}$ wing discs is up-regulated in comparison with the expression of β–GAL in a control *sdGal4>diap1-lacZ* wild type discs (compare Figures 23A and B), suggesting that inactivation of *skpA*-induced apoptosis affects *diap1* transcription. Next, we studied whether the expression of DIAP1 protein was induced by inactivation of *skpA*. For that we stained with anti-DIAP1 antibody (Ryoo et al., 2002) and analyzed DIAP1 expression in *enGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* wing imaginal discs, where *skpA* had been inactivated in the posterior compartment. Figure 23C shows that the inactivation of *skpA* decreases the levels of DIAP1 protein, suggesting that the apoptosis induced by loss of *skpA* function down-regulates DIAP1 protein levels.

Hence, the analysis of the regulation of *diap1* expression indicates that inactivation of *skpA* induces *diap1* transcription and decreases DIAP1 protein levels, suggesting that down-regulation of DIAP1 protein occurs through a post-transcriptional mechanism.

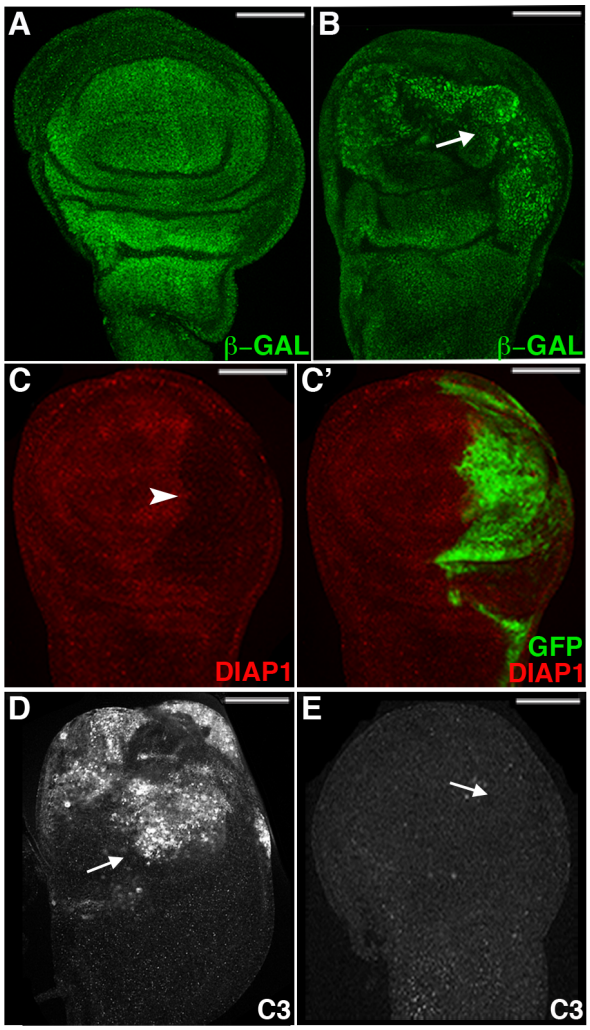


Figure 23. Inactivation of *skpA* promotes *diap1* transcription and decreases DIAP1 protein levels. (**A**) Wing disc *diap1-lacZ* showing β-GAL (green) expression. (**B**) Wing disc *sdGal4>diap1-lacZ/UAS-skpA*$_{RNAi}$ showing β-GAL (green) expression (arrow). Note the increase in β-GAL expression in the cells where *skpA* has been inactivated (arrow). (**C**) Wing disc *enGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing DIAP1 (red) expression. Note the decrease of DIAP1 protein levels due to the inactivation of *skpA* in the posterior compartment (arrowhead). (**C'**) Merged image of (C) and GFP (green) expression. (**D**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing C3 (white) expression (arrow). (**E**) Wing disc *sdGal4>UAS-DIAP1;UAS-skpA*$_{RNAi}$ showing C3 (white) expression. Note that the number of apoptotic cells decreases compared to (D) (arrow). Scale bars represent 100μm

3.5.5. Inactivation of *skpA*-induced apoptosis is dependent on DIAP1 expression

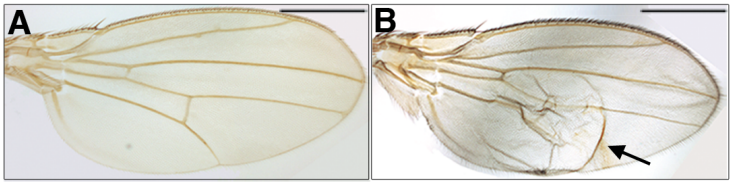
Next, we studied the effect of DIAP1 overexpression in the apoptosis mediated by inactivation of *skpA*. For that we analyzed C3 expression in wing discs were DIAP1 was overexpressed and *skpA* simultaneously inactivated (*sdGal4>UAS-DIAP1;UAS-skpA*$_{RNAi}$) and compared it to C3 expression in wing discs where we only *skpA* was inactivated (*sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP*). We found that expression of C3 in *sdGal4>UAS-DIAP1;UAS-skpA*$_{RNAi}$ wing imaginal discs (Figure 23E) is decreased when compared to *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* wing imaginal discs (Figure 23D). Thus, these results show that high levels of DIAP1 notably reduce the number of C3 expressing cells due to the inactivation of *skpA* function, indicating that the apoptosis induced by loss of *skpA* function is dependent on DIAP1 levels.

3.5.6. Inactivation of *dRYBP*-induced apoptosis is dependent on *rpr* and DIAP1 expression

We next studied whether dRYBP could be regulating *rpr* transcription and Rpr protein levels using S2 cells. First we inactivated *dRYBP* in S2 cells using the dsRNA generated for this thesis (dRYBP KD) and then measured the levels of mRNA of the *rpr* and *hid* genes by RT-qPCR (Figure 21D). The results show that there are not significant variations in the levels of mRNA of *rpr* nor *hid* between wild type S2 cells (Mock) and dRYBP KD S2 cells, suggesting that dRYBP does not regulate the transcription of either *rpr* nor *hid* (Figure 21D). Furthermore, protein extracts from dRYBP KD S2 cells were resolved by SDS-PAGE and analyzed by WB with anti-Rpr antibody (a gift from H. Steller, unpublished). Figure 21E shows that the inactivation of *dRYBP* does not have an effect on the levels of Rpr protein. These results indicate that *dRYBP* loss of function does not regulate *rpr* transcription or protein levels. Nevertheless these undetectable effects on *rpr* expression may be due to the low levels of apoptosis observed when *dRYBP* is inactivated (Figure 19A).

Furthermore we studied whether the inactivation of *rpr* function and the overexpression of DIAP1 had an effect on the inactivation of *dRYBP*-induced apoptosis (Figure 24). The expression of C3 in *enGal4>UAS-dRYBP*$_{RNAi}$*/UAS-GFP* wing imaginal discs is only observed in a low number of cells (Figure 19F). Therefore to study if the apoptosis induced by *dRYBP* loss of function was dependent on *rpr* expression and DIAP1 protein levels, we analyzed the modulation of the penetrance of the blistered wing phenotype due to the inactivation of *dRYBP*, previously associated to apoptosis (Dichtel-Danjoy et al., 2013; Gonzalez et al., 2008). The results show that 70% of the flies *enGal4>UAS-dRYBP*$_{RNAi}$*/UAS-GFP*, where *dRYBP* has been inactivated present blistered wings (Figures 24B and C, (Gonzalez et al., 2008)) while none of the *enGal4>UAS-dRYBP*$_{RNAi}$*/UAS-rpr*$_{RNAi}$ flies, where *dRYBP* and *rpr* have been simultaneously inactivated show blistered wings (Figure 24C). This result suggests that the apoptosis induced

by the loss of *dRYBP* function is dependent on *rpr* levels of expression. Furthermore, the penetrance of the wing blistered phenotype is also reduced (24%) in *enGal4>UAS-DIAP1;UAS-dRYBP$_{RNAi}$* flies, where DIAP1 has been overexpressed and *dRYBP* has been simultaneously inactivated (Figure 24C), indicating as well, that *dRYBP* inactivation-induced apoptosis is mediated by DIAP1 protein.



C Genotype	Blisters (%)	n
en>rpr$_{RNAi}$/GFP	0	98
en>dRYBP$_{RNAi}$/GFP	70	100
en>dRYBP$_{RNAi}$/rpr$_{RNAi}$	0	100
en>DIAP1;dRYBP$_{RNAi}$	24	94

Figure 24. Apoptosis induced by the inactivation of *dRYBP* is dependent on levels of Rpr and DIAP1. (**A**) Wild type wing (**B**) Wing *enGal4>UAS-dRYBP$_{RNAi}$* showing blister in the posterior compartment due to *dRYBP* inactivation (arrow). Scale bars represent 0.5mm. (**C**) Penetrance of the blistered wing phenotype in the corresponding genotypes. Table shows the percentage of flies with blistered wings. Note that inactivation of *rpr* and overexpression of DIAP1 together with *dRYBP* inactivation reduce the percentage of flies with wing blisters in comparison to flies where only *dRYBP* has been inactivated.

When considered together, the results from the epistatic relationships between *dRYBP* and *skpA* with *rpr* and *DIAP1* indicate that the dRYBP-SCF complex functions to inhibit the intrinsic apoptotic pathway. Moreover, the results also indicate that the inhibition of apoptosis takes place at the level of Rpr and DIAP1 proteins.

3.6. SKPA protein biochemically interacts with Rpr and DIAP1 proteins

To study the functionality of the regulation of the intrinsic apoptotic pathway by the dRYBP-SCF complex we examined the interactions between SKPA, Rpr and DIAP1.

To analyze the biochemical interaction between SKPA, Rpr and DIAP1 we used HEK293 (Human embryonic kidney) cells, a line that shows high transfection efficiency. To study the interaction between SKPA and DIAP1 we generated an skpA-FLAG plasmid that was co-transfected with DIAP1-GST (Sandu et al., 2010) in HEK293 cells. Moreover, to study the interaction between SKPA and Rpr we co-transfected with skpA-FLAG and rpr-HA (Sandu et al., 2010) HEK293 cells. Additionally, cells were transfected with each vector independently as controls. Cell extracts were then used to perform Co-IPs using anti-FLAG beads. The eluted

proteins were then resolved by SDS-PAGE, followed by WB analysis with the corresponding antibodies (Figure 25). The Co-IPs experiments show that SKPA interacts with DIAP1 protein as well as with Rpr protein, indicating that SKPA biochemically interacts with DIAP1 and Rpr (Figure 25). Notably Rpr protein levels are highly reduced in HEK293 cells co-transfected with skpA-FLAG and rpr-HA when compared to Rpr protein levels in HEK293 cells only transfected with rpr-HA, suggesting that high levels of SKPA protein reduces levels of Rpr protein (Figure 25B).

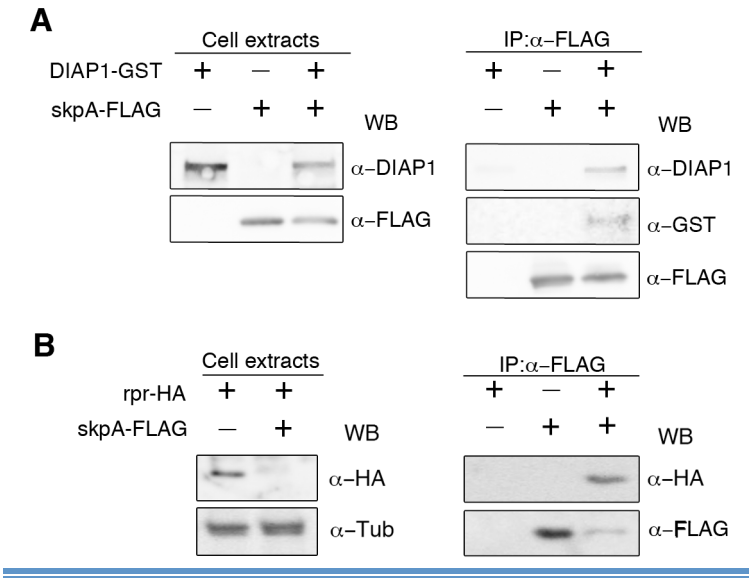


Figure 25. SKPA biochemically interacts with DIAP1 and Rpr. (**A**) HEK293 cell extracts were transfected with skpA-FLAG or DIAP1-GST or simultaneously co-transfected. Cell extracts were IP using α–FLAG-beads (IP: α–FLAG). Precipitates were analyzed by WB using antibodies against DIAP1, GST and FLAG. (**B**) HEK293 cells were transfected with skpA-FLAG or rpr-HA or simultaneously co-transfected. Cell extracts were IP using α–FLAG-beads (IP:α–FLAG). The precipitates were analyzed by WB using antibodies against HA, FLAG and Tubulin. Note levels of HA (indicative of Rpr) decrease in cell extracts co-transfected with skpA-FLAG+rpr-HA in comparison to HA levels in cell extracts transfected with rpr-HA.

This result is in accordance with the results obtained in the wing, where high levels of SKPA rescue the Rpr overexpression wing phenotype (Figure 22E), suggesting once more that SKPA inhibits apoptosis through the regulation of Rpr protein levels.

3.7. High levels of SKPA protein inhibit developmental and stress-induced apoptosis

To understand the mechanism of the dRYBP-SCF complex in the inhibition of apoptosis, we investigated whether high levels of these proteins where able to inhibit developmental apoptosis, a process required to achieve proper leg morphogenesis (Fuchs and Steller, 2011; Manjon et al., 2007). In addition we studied if the dRYBP-SCF complex could repress the

radiation-induced apoptosis, associated with the elimination of damaged cells (Wichmann et al., 2006). We chose to use the overexpression of SKPA for these experiments because overexpression of Slmb does not produce any visible phenotype, and high levels of either dRYBP (Gonzalez and Busturia, 2009) or dCUL1 both induce apoptosis (Appendix 6).

The distal part of the legs is composed of five tarsus (T1-T5, Figure 26A) separated by joints. The morphogenesis of joints requires the activation of cell death in specific tarsus cells (Manjon et al., 2007). SKPA was overexpressed using either *Distal-less-Gal4* (*DllGal4*) or *rotund-Gal4* (*rnGal4*) lines that drive expression in the distal part of the leg (Estella et al., 2008; Kerridge, 1988). The results show that high levels of SKPA protein interferes with leg joint formation both in *rnGal4>UAS-skpA*^{OB} (Figures 26B and F) and *DllGal4>UAS-skpA*^{OB} (Figures 26D and H). In addition, we analyzed whether this effect was mediated by the repression of the intrinsic pathway of apoptosis by studying the expression of *rpr-lacZ* (Figures 26I-L), as a readout of apoptosis, in *rnGal4>rpr-lacZ;UAS-skpA*^{OB} leg imaginal discs. The results show that *rpr-lacZ* expression (Figures 26I and K) is significantly ($p<0.005$) decreased by 33% (Figure 26M) in the leg imaginal discs due to the high levels of SKPA expression both in the prothoracic (Figure 26J) and the metathoracic (Figure 26L) leg discs. These results indicate that *skpA* functions to inhibit developmental apoptosis by interfering with the intrinsic apoptotic pathway.

We next analyzed whether high levels of SKPA are capable of inhibiting X-ray-induced apoptosis. For that we irradiated *hhGal4>UAS-skpA*^{OB}*/UAS-GFP* larvae and then analyzed, quantified and compared C3 expression in the anterior and posterior compartments of wing imaginal discs (Figures 26N-O). The results show that C3 expression in the posterior compartment of *hhGal4>UAS-skpA*^{OB}*/UAS-GFP* wing discs, where SKPA has been overexpressed, is significantly ($p<0.001$) reduced by 20% in comparison to the wild type anterior compartment (Figures 26N-O).

Taken together, our results show that high levels of SKPA are capable of inhibiting both the developmental- and X-ray-induced apoptosis and provide further evidence that the dRYBP-SCF complex functions as survival factors in these processes.

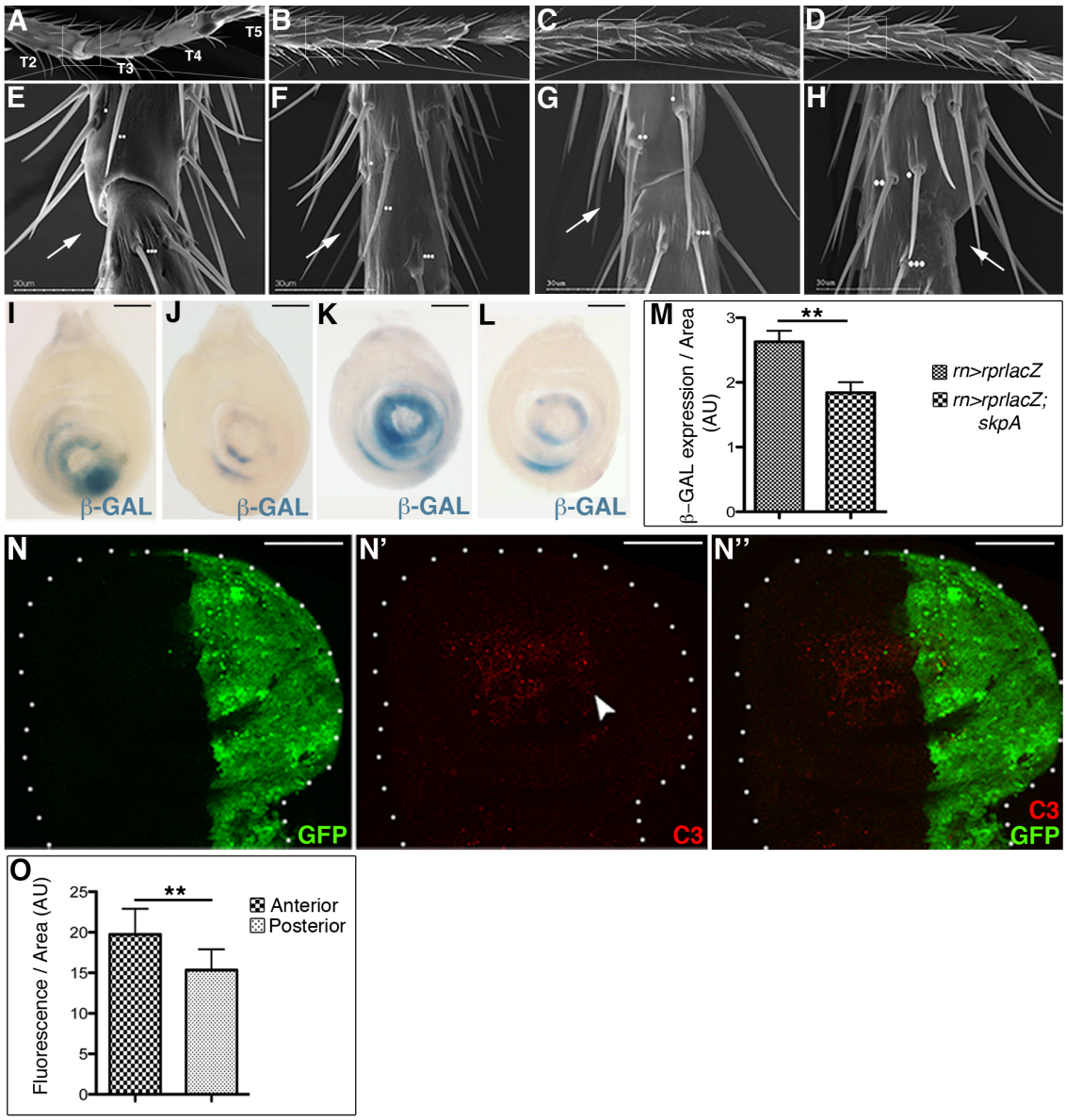


Figure 26. High levels of SKPA inhibit developmental and X-Ray-induced apoptosis. (**A-D**) Distal regions of prothoracic legs showing tarsal segments T2-T5. Squares include the joint between T2 and T3. (**A**) *rnGal4>UAS-GFP*. (**B**) *rnGal4>UAS-skpA*^{OB}. (**C**) *dllGal4>UAS-GFP*. (**D**) *dllGal4>UASskpA*^{OB}. (**E-H**) Enlargement of area in the squares indicated in (A), (B), (C) and (D) showing joint between T2 and T3. White dots indicate position of corresponding bristles as reference points to locate the joints (arrows) in the legs of the different genotypes. (**E**) Enlargement of (A). (**F**) Enlargement of (B). Note the absence of joint between T2 and T3 compared to (E). (**G**) Enlargement of (C). (**H**) Enlargement of (D). Note That the joint (arrow) is not properly formed compared to (G) (arrow). Scale bars represent 30μm. (**I-J**) Prothoracic leg disc *rnGal4>rpr-lacZ;UAS-GFP* (**I**) and *rnGal4>rpr-lacZ;UAS-skpA*^{OB} (**J**) showing β-GAL (blue) expression. Note decreased levels of β-GAL expression in (J) compared to (I). (**K-L**) Metathoracic leg disc *rnGal4>/rpr-lacZ;UAS-GFP* (**K**) and *rnGal4>/rpr-lacZ;UAS-skpA*^{OB} (**L**) showing β-GAL (blue) expression. Note the decrease of β-GAL expression in (L) compared to (K). Scale bars represent 50μm. (**M**) β-GAL expression quantification (fluorescence/area) in the indicated genotypes (n=25). Error bars represent SEM and asterisks the following p values: ** p<0.005. AU= Arbitrary Units. (**N-N''**) Irradiated wing disc *hhGal4>UAS-skpA*^{OB}*/UAS-GFP* showing GFP (green) (**N**) expression, labeling the *hhGal4* domain in the posterior compartment, C3 (red) (**N'**) expression and merged image (**N''**). Note the reduction of apoptotic cells (arrowhead) in the posterior compartment due to the high levels of SKPA. Scale bars represent 100 μm. (**O**) C3 expression quantification (fluorescence/area) in the anterior and posterior compartment of wing discs *hhGal4>UAS-skpA*^{OB}*/UAS-GFP* irradiated at 2000R and dissected 24h after irradiation (n=16). Note C3 fluorescence/area is significantly reduced in the posterior compartment due to SKPA overexpression. Error bars represent SEM and asterisks denote the following p values: ** p<0.001. AU= Arbitrary Units

Discussion

1. dRYBP IS A UBIQUITIN BINDING PROTEIN

Ubiquitylation is a post-translational modification that results in the covalent attachment of one or several ubiquitin molecules to a lysine residue in the target protein (Ciechanover et al., 1980; Hershko et al., 1979). The physiological outcome of protein ubiquitylation can vary, depending on the number of ubiquitin molecules attached and the type of linkage to one another from DNA repair, epigenetic regulation of gene expression, protein degradation, endocytosis or other functions (Chau et al., 1989; Chen and Sun, 2009). The ubiquitylation pathway regulates biological processes of great relevance, such as cell death (Bergmann, 2010; Tan et al., 2006), cell cycle (Lau et al., 2012) and the immune response (Zinngrebe et al., 2014), and has to be strictly regulated to assure its specificity for the target protein, as well as specify the number of ubiquitin molecules and how ubiquitin is attached to target proteins. Miss-regulation of ubiquitylation has been implicated in the pathogenesis of multiple diseases such as Parkinson's and cancer (Inuzuka et al., 2011; Thompson et al., 2008); therefore targeting the ubiquitylation pathway is being actively investigated for discovery and drug development (Zhang and Sidhu, 2014).

Once a protein is ubiquitylated, the modification has to be recognized so the downstream biochemical reactions take place. Some of the mechanisms involving recognition of the specific ubiquitylated target require a ubiquitin-binding protein, which contains in its sequence a UBD (ubiquitin binding domain). UBDs are protein domains that non-covalently bind mono- or polyubiquitin chains and can be post-translationally modified by ubiquitylation. They are structurally diverse and have a range of binding affinities to ubiquitin. Moreover, different type of UBDs can recognize distinct types of ubiquitin modifications and are present in proteins that have different biological functions (Hicke et al., 2005) (Table 1). For instance, some of the UBDs that have been characterized so far are the UBA (ubiquitin associated domain), which is present in enzymes involved in the catalytic reactions of ubiquitylation and de-ubiquitylation (Hofmann and Bucher, 1996), the UIM (ubiquitin-interacting motif), present in the subunit of the proteasome S5a/Rpn10 responsible for proteasomal degradation (Young et al., 1998) and the NZF (Nucleoporin Zinc finger), present in proteins involved in different biological processes such as the immune response (Kanayama et al., 2004) and endocytosis (Alam et al., 2004).

In this thesis work, we have discovered that the dRYBP protein coexists in S2 cells in two different forms, dRYBP and dRYBPub (Figure 9A). Consistent with this data, the UBD contained in RYBP protein, the vertebrate homolog of dRYBP, has also been found to be monoubiquitylated (Alam et al., 2004; Arrigoni et al., 2006), reinforcing the functional homology between these two proteins. The different functions of dRYBP and the dRYBPub form remain to be determined. Our results show that dRYBP monoubiquitylation is not required for its degradation via proteoasome (Figure 9A). This result is not surprising as protein

monoubiquitylation has been shown to have different roles from proteasomal degradation (Chau et al., 1989; Hoeller et al., 2006). Our results indicate that the dRYBP form interacts with the PcG and trxG proteins and with the SCF complex components (Figures 10 and 15) (discussed in sections 2 and 3). Perhaps the dRYBPub form affects its functionality, as it has been shown that monoubiquitylation of UBD-containing proteins can have a negative regulatory role, by promoting protein-protein interactions that prevent recognition of ubiquitylated targets (Hoeller et al., 2006). Alternatively monoubiquitylation of UBD-containing proteins can mediate signal amplification, also generated by protein interactions, or promote membrane fusion leading to vesicle formation and endocytosis (Alam et al., 2004; Hicke et al., 2005). Perhaps monoubiquitylation of dRYBP may be involved in the shuttling from the nucleus to the cytoplasm and could help explain the role of this protein in the immune response (Aparicio et al., 2013) and in apoptosis, described in this thesis work (Section 3 of the results).

We have also found that dRYBP interacts with ubiquitylated proteins mainly through its NZF domain (Figure 9C). Our results shown that the dRYBPΔNZF construct, lacking the UBD, very poorly binds to ubiquitylated proteins in comparison to the dRYBP full length protein which binds numerous (Figure 9C), indicating that the UBD is indeed involved in the interaction with ubiquitylated proteins. Curiously, the truncated version of the dRYBP protein lacking its UBD, dRYBPΔNZF can also interact with histones H2A, H2Aub, H2B and H2Bub (Figure 9D). This result indicates that the NZF domain of the dRYBP protein is not required for H2Aub and H2Bub binding, although we cannot discard this domain enhances these interactions. Perhaps dRYBP binding to histones may be an indirect consequence of its interaction with PcG and trxG proteins (discussed in section 2).

Taken together, our results suggest that dRYBP could be functioning as a ubiquitin adaptor protein to promote the assembly of different ubiquitylated proteins or to activate signaling pathways as previously described for other UBD-containing proteins. For instance, binding of TAB2 and TAB3 (both NZF-containing proteins) to –K63 linked polyubiquitylated proteins is required for the activation of the NF-kb signaling pathway (Kanayama et al., 2004). Also binding of the Vsp36 protein (NZF-containing protein) to ubiquitylated proteins triggers the endosomal pathway activation (Alam et al., 2004; Hicke et al., 2005).

We propose that in similar way to other NZF-containing proteins dRYBP binds to ubiquitylated proteins through its NZF to promote protein-protein interactions leading to the activation of a signaling cascade, such as apoptosis (Figure 27A).

This would help explain why dRYBP is able to interact with a wide variety of proteins including transcription factors (Schlisio et al., 2002), apoptotic-related factors (Gonzalez and Busturia, 2009) and also, the diversity of biological processes that dRYBP is involved in such as the immune response, apoptosis and morphogenesis (Aparicio et al., 2013; Gonzalez et al., 2008; Gonzalez and Busturia, 2009; Novak and Phillips, 2008). Interestingly the NZF is not essential for the binding to H2A, H2Aub, H2B and H2Bub therefore it is to be determined whether this putative function as a ubiquitin adaptor protein is achieved by its NZF domain (Figure 27).

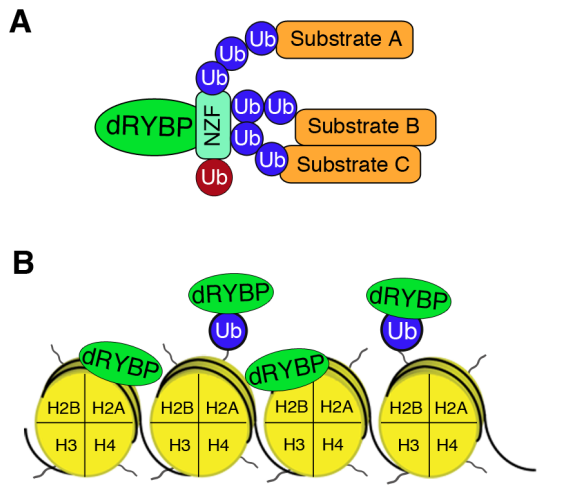


Figure 27. dRYBP binds ubiquitylated proteins and histones H2A, H2Aub, H2B and H2Bub. (**A**). Representation of the ubiquitin adaptor function of dRYBP. The dRYBP protein can bind ubiquitin (red) and ubiquitylated proteins through its NZF domain. (**B**) Representation of the nucleosomes formed by the histones H2A, H2B, H3 and H4. The dRYBP protein can bind directly or indirectly to histones H2A and H2B and their modified version H2Aub and H2Bub.

2. dRYBP INTERACTS WITH PcG AND trxG PROTEINS AND MODULATES LEVELS OF HISTONE POST-TRANSLATIONAL MODIFICATIONS

The PcG and trxG proteins are responsible for the maintenance of the transcriptional repressed and activated states respectively of target genes throughout development and during the adult life (Schuettengruber and Cavalli, 2010; Schwartz and Pirrotta, 2007). These proteins form multimeric protein complexes with a different biochemical activity (Table 1 and 2), such as the repressor complex PRC1 (formed by the proteins SCE/dRING, PSC, PC and PH) responsible for ubiquitylation of H2AK119 (H2AK118 in *Drosophila*) (Endoh et al., 2012; Gutierrez et al., 2012; Shao et al., 1999) or the activator macrocomplex COMPASS, (that comprise the Trx-, Trr-, dSet- and Ash1-COMPASS complexes) responsible for mono-, di- and trimethylation of H3K4 (Mohan et al., 2011). However, the components of each complex can associate to other proteins leading to variants of the canonical complexes and consequently vary its function. For example, the dRAF complex (composed of SCE/dRING, PSC and dKDM2) is a variant of the PRC1 complex that represses transcription by more efficiently ubiquitylating H2A than PRC1 and by demethylating H3K36me2 (Lagarou et al., 2008). In this thesis work we have discovered the association of dRYBP with both repressor and activator complexes and the functional consequences of these interactions as discuss below.

2.1. dRYBP interactions define novel functional repressor and activating protein complexes

Mass spectromery analysis performed in the laboratory (Simón, R. *et al*, manuscript in preparation) (Simón, 2013) using anti-dRYBP antibody (Bejarano et al., 2005) in wild type dNE pointed out that dRYBP interacts with members from the PcG and from the trxG group of proteins (Table 12), consistent with previous results from the laboratory (Bejarano et al., 2005; Gonzalez et al., 2008). Because of the findings in this thesis work, the nature of dRYBP as a ubiquitin binding protein and its capability of interacting with H2Aub and H2Bub (Figure 9), we selected among the different proteins shown in the mass spec analysis to study the interaction of dRYBP with SCE/dRING and dKDM2, responsible for H2Aub (Lagarou et al., 2008) and with dBRE1, responsible for H2Bub (Hwang et al., 2003).

We confirmed through Co-immunoprecipitation experiments that dRYBP binds to SCE/dRING, dKDM2 and dBRE1 (Figure 10) and that it does not interact with PSC, PC, PH and EZ, none of which appeared as dRYBP interactors in the mass-spec data (Simón, R. *et al*, manuscript in preparation) (Simón, 2013). Recent studies in vertebrates have shown that distinct subunits of PCGF (PSC homologues) can form PRC1 variants through their interaction with RYBP (dRYBP homolog) and also that this association prevents the incorporation of other PRC1 members, such as the CBX (PC homolog), PHC (PH homolog) and SCML (SCM homolog) proteins, identifying two PRC1 variants: a) PRC1-CBX, the canonical PRC1 containing CBX protein and b) PRC1-RYBP, non canonical PRC1 containing RYBP protein (Gao et al., 2012; Morey et al., 2013; Tavares et al., 2012). Our results show that dRYBP does not interact with the PRC1 members PC and PH, suggesting it forms an alternative complex to the canonical PRC1. However, we have shown that dRYBP only interacts with one of the PRC1 components, the SCE/dRING protein, suggesting that the composition of the different PcG complexes is not conserved from fly to vertebrates. Curiously, we have shown that dRYBP and PSC do not biochemically interact (Figures 10A and C) and since dRYBP binds to SCE/dRING and dKDM2 but not to PSC, all three members of the dRAF complex, our finding suggests that dRYBP could be forming and alternative complex to dRAF with SCE/dRING and dKDM2 excluding PSC that we have named dRRK (dRING, dRYBP and dKDM2) complex. We still do not know the relevance of the absence of PSC, an E3 ligase that promotes H2Aub and also stimulates the H2Aub activity of the SCE/dRING (Buchwald et al., 2006; Cao et al., 2005; Lagarou et al., 2008), in the putative dRRK complex but it will be discussed below in section 2.3. Interestingly the dRAF complex is considered to be the homolog of the mammalian BCOR complex (Huynh et al., 2000; Lagarou et al., 2008), which contains the RYBP and BMI-1 (PSC in *Drosophila*) proteins. However, our results show that this is not the case (Figure 10) further

indicating that the composition of the dRYBP-containing complexes does not seem to be conserved between *Drosophila* and vertebrates.

Furthermore, we have shown that dBRE1 biochemically interacts with dRYBP but not with SCE/dRING and dKDM2 (Figure 10C), suggesting the existence of a dRYBP-dBRE1 (dRB) complex independent from other PcG proteins. Moreover, dRYBP did not interact in the mass spectrometry analysis with Rad6 protein, the E2 conjugating enzyme that interacts with dBRE1 to ubiquitylate H2B (Hwang et al., 2003; Kao et al., 2004; Robzyk et al., 2000). Perhaps the dRYBP protein impedes Rad6 from interacting with dBRE1 protein, as discussed below in section 2.3.

Interestingly the pulldown assay shows that the association of dRYBP to SCE/dRING, dKDM2 and dBRE1 does not require the NZF domain (Figure 10D). This suggests that a different specific domain is responsible for this interaction, however we cannot discard that the NZF may enhance the interaction between dRYBP and the SCE/dRING, dKDM2 and dBRE1 proteins. Moreover the association of dRYBP to PcG and trxG may indirectly mediate its binding to H2A, H2Aub, H2B and H2Bub. *In vitro* binding experiments should be carried out to investigate what dRYBP-domain is necessary for the binding to PcG and trxG proteins and to histones, and also to study if the binding to histones is direct or indirect.

2.2. dRYBP modulates levels of histone post-translational modifications

Post-translational modifications of histones are responsible for chromatin remodeling and therefore, are ultimately responsible for gene transcriptional repressed and activated states. Among the better-characterized histone post-translational modifications and of interest for this thesis are the monoubiquitylation of H2A (H2Aub) the mono-, di- and trimethylation of H3K36 and the monoubiquitylation of H2B (Henry et al., 2003; Muller et al., 2002; Shilatifard, 2006; Wang et al., 2004).

SCE/dRING protein is the major E3 ubiquitin ligase responsible for H2Aub (Buchwald et al., 2006; Gutierrez et al., 2012). Our results demonstrate that the inactivation of *dRYBP* in S2 cells decreases the levels of H2Aub (Figures 12 and 14A) indicating that dRYBP promotes H2Aub, a landmark for gene silencing (Wang et al., 2004). This result together with the results from the genetic interactions, which show that that *dRYBP* enhances the repressor effect of *Sce/dRing* (Figure 11F), indicate that dRYBP is required for gene repression. We have shown that dRYBP binds to H2A and H2Aub (Figure 9D), yet dRYBP does not contain a ubiquitylating catalytic domain, therefore we propose that the role of dRYBP in H2Aub may be through its interaction with SCE/dRING by enhancing its E3 ubiquitin ligase activity. We could not test this possibility as the inactivation of *Sce/dRing* already shows a nearly complete

depletion of H2Aub (Figure 14A) and an increase in the reduction of H2Aub levels when *dRYBP* was simultaneously inactivated was very difficult to appreciate (Figure 14A). Accordingly, recent data has shown that RYBP is an essential cofactor required for ubiquitylation of H2A by RING1B as its inactivation reduces levels of H2Aub, supporting the role of dRYBP in gene repression through H2Aub (Tavares et al., 2012).

Furthermore, previous results show that the dKDM2 protein enhances transcriptional repression by promoting H2Aub by dRING, a mark for gene repression, and by H3K36me2 demethylation, counteracting the activating mark established by trxG (Lagarou et al., 2008). These biochemical activities of dKDM2 lead to a decrease in levels of H2Aub and to an increase of H3K36me2 when *dkdm2* is inactivated ((Lagarou et al., 2008), Figure 14B). However when *dRYBP* is inactivated the levels of H3K36me2 are not affected (Figure 12). Interestingly, the levels of H3K36me2 are reduced when *dRYBP* and *dkdm2* are simultaneously inactivated in comparison to the high levels of H3K36me2 when *dkdm2* is inactivated (Figure 14B). This result is consistent with the genetic results presented in this work showing that *dRYBP* counteracts *dkdm2* function in the control of homeotic gene expression (Figures 11E and F).

Taken together our results suggest that dRYBP functions to counteract the repressor effects mediated by dKDM2, but the function of dRYBP in H3K36me2 demethylation while biochemically interacting with dKDM2 remains to be determined. It is known that H2Aub and H3K36me2 demethylation are two independent biochemical reactions (Lagarou et al., 2008). Therefore we propose that the physical interaction between dRYBP and dKDM2 is what counteracts the repressor effect of dKDM2. Perhaps, the binding of dRYBP protein to dKDM2 blocks or changes the conformation of its JmjC domain, required for H3K36me2 demethylation activity (Klose et al., 2006; Tsukada et al., 2006), and impedes dKDM2 to efficiently demethylate H3K36me2. Further studies should be carried out to clarify the function of dRYBP on the H3K36me2 demethylation mediated by dKDM2.

On the other hand, we have shown that the inactivation of *dRYBP* affects the levels of H3K4me, a landmark responsible for gene activation, indicating the role of dRYBP in transcriptional gene activation. dRYBP did not interact in the mass spectrometry analysis with the Trr protein, responsible for H3K4me (Herz et al., 2013), however it interacts with other components of the dCOMPASS complexes, thus this putative interactions should be analyzed to help us understand the dRYBP function in H3K4me.

Finally, we have shown that *dBre1* inactivation induces A5 transformation to A4, a *trxG* mutant-associated phenotype. This homeotic phenotype, here described for the firs time, in *dBre1* homozygous mutants indicates that dBRE1 should be considered as a trxG member.

Additionally, we have demonstrated that *dRYBP* inactivation does not vary levels of H2Bub (Figure 12). However, when *dRYBP* is concomitantly inactivated with *dBre1*, the levels of H2Bub are increased in comparison to the levels of H2Bub in *dBre1* inactivation (Figure 14C). Thus dRYBP is able to partially restore the levels of H2Bub indicating that dRYBP counteracts dBRE1 function and this is consistent with the genetic results presented in this work indicating that *dRYBP* suppresses the activator effect of *dBre1* (Figure 11G). It remains unclear how dRYBP can counteract dBRE1 function. Perhaps the interaction of dRYBP protein with dBRE1 blocks partially the RING domain of dBRE1 preventing it from efficiently ubiquitylating H2B. Alternatively, the binding of dRYBP to H2B and H2Bub (Figure 9D) might be what inhibits dBRE1 E3 ubiquitin ligase activity, suggesting in this case that the role of dRYBP as a ubiquitin binding protein would be to impede dBRE1 from ubiquitylating H2B.

Taken together, the results from the genetic interactions and the analysis of the modulations of levels of post-translational histone modifications suggest that dRYBP is responsible for regulating gene transcriptional repression and gene transcriptional activation.

2.3. A model for dRYBP function in the epigenetic regulation of gene expression

The results from the analyses of the dRYBP-biochemical interactions, the genetic studies and the investigations of the effects on the levels of histone post-translational modifications reveal the modulation of repressed and activated transcriptional states mediated by dRYBP protein. These findings have made us propose a model whereby dRYBP is involved in the epigenetic regulation of gene expression by promoting intermediate levels of gene transcriptional repression and gene transcriptional activation (Figure 28). In this scenario dRYBP would alleviate the repression executed by the dRAF complex and also the activation executed by the dBRE1 protein.

In this model dRYBP binds to SCE/dRING and dKDM2 proteins, both members of the dRAF complex, excluding PSC to form the dRRK complex (Figure 28), as dRYBP does not biochemically interact with PSC (Figure 10). The biochemical function of this complex would be to promote ubiquitylation of H2A, as we an others have shown inactivation of each component decreases levels of H2Aub (Figure 14A, (Lagarou et al., 2008; Tavares et al., 2012)). Ubiquitylation of H2A by the dRRK complex could be either by enhancing the E3 ubiquitin ligase of SCE/dRING and/or by the ubiquitin adaptor function of dRYBP, which would recruit the dRRK complex to the H2A leading to H2Aub. However, it would not reach the levels of H2Aub mediated by dRAF, as dRYBP binding excludes PSC, an E3 ubiquitin ligase that enhances but also ubiquitylates H2A itself. Furthermore, the interaction between dRYBP and dKDM2 would impede dKDM2 to efficiently demethylate H3K36me2. Therefore

the dRRK complex would be unable to effectively repress gene expression both by its effects on H2Aub and H3K36me2. This would generate an intermediate state of gene transcriptional repression where there are high levels of H2Aub and intermediate levels of H3K36me2 in comparison to the efficient gene repression generated by the dRAF complex, where there are high levels of H2Aub and high levels of demethylated H3K36me2 (H3K36me) (Figure 28).

On the other hand, we propose that dRYBP protein would bind to dBRE1 to form a different transcriptional activator complex as these two proteins biochemically interact independently from other PcG proteins (Figure 10). In this case either the physical interaction of dRYBP and dBRE1 or the interaction between dRYBP and H2B would lead to an inefficient H2Bub by dBRE1 (Figure 28), a histone landmark required for the recruitment of other transcriptional activators (Nakanishi et al., 2009; Sun and Allis, 2002). This is supported by the genetic interaction between *dRYBP* and *dBre1* and the modulation of H2Bub levels when we simultaneously inactivate *dRYBP* and *dBre1* (Figures 11G and 14C). Thus we propose that the dRYBP-dBRE1 complex (dRB) would generate an intermediate state of gene activation where there are intermediate levels of H2Bub and cannot efficiently recruit the COMPASS complexes, in comparison to a state of gene activation where dBRE1 does not interact with dRYBP and is able to efficiently ubiquitylate H2B, consequently recruiting the COMPASS complexes to di- and trimethylate H3K4 contributing to transcriptional activation (Figure 28).

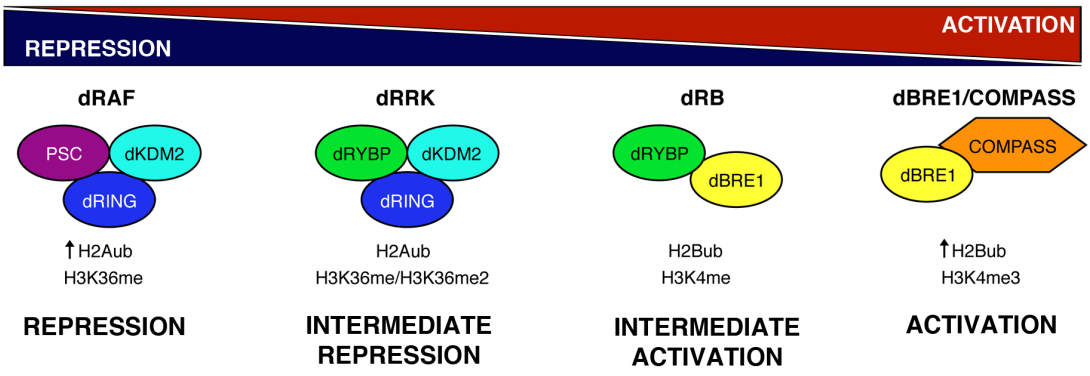


Figure 28. Proposed model for the function of dRYBP in epigenetic regulation. dRAF complex formed by the proteins PSC, dRING and dKDM2 generate transcriptional repressed states by ubiquitylation of H2A, leading to high levels of H2Aub (indicated with an arrow) and by demethylation of H3K36me2, leading to high levels of H3K36me. dRRK formed by dRYBP, dRING and dKDM2 also generate transcriptional repressed states by promoting H2Aub. However the absence of PSC, an H2A E3 ubiquityl ligase, decreases the levels of H2Aub and the interaction of dRYBP and dKDM2 leads to an inefficient demethylation of H3K36me2, leading to intermediate state of gene repression with lower levels of H2Aub and intermediate states of H3K36me2 and H3K36me. dRB complex is formed by the dRYBP and dBRE1 proteins. This complex inefficiently ubiquitylates H2B (H2Bub) and consequently cannot efficiently recruit COMPASS complexes to di- and tri-methylate H3K4, generating an intermediate state of gene activation. The absence of dRYBP promotes an efficient H2Bub by dBRE1 (indicated with an arrow), which recruits the COMPASS complexes, leading to state of gene activation with high levels of H2Bub and trimethylated H3K4 (H3K4me3).

Our findings reveal for the first time, that the interaction of dRYBP with SCE/dRING and dKDM2 promotes attenuation of the levels histone modifications associated with gene repression and also that the interaction of dRYBP with dBRE1 promotes attenuation of the levels histone modifications associated with gene activation. This strongly suggests that the presence of dRYBP in these complexes is required to promote intermediate levels of gene expression. This is the first time that an ETP protein has been shown to have a direct function in post-translational modification of histones, in particular by modulating repressed and activated gene transcriptional states. This thesis work points out the relevance of studying epigenetic regulation by ETPs, as perhaps the dRYBP function could be extended to other ETPs and could help understand the plasticity of epigenetic regulation of gene expression.

Moreover this thesis work indicates the relevance of intermediate states of gene expression. For instance, the homeotic gene *Ubx* is differentially expressed in the *Drosophila* embryo, in the PS4 it is repressed, in the PS5 it shows intermediate levels of expression and in the PS6 it is activated. We propose the dRYBP is responsible for the intermediate repressive and activating transcriptional states and is a key balancer in the regulation mediated by PcG and trxG. Supporting this idea, mammalian PRC1 RYBP-containing complexes are proposed to mediate low gene repression opposed to other PRC1 non RYBP-containing complexes that promote high gene repression (Morey et al., 2013). Further analysis should be carried out to validate this model, which would help us understand the plasticity and the capability of transcription-dependent functions of PcG and trxG proteins in normal and pathological development.

3. A NOVEL dRYBP-SCF COMPLEX FUNCTIONS TO INHIBIT APOPTOSIS

Programmed cell death is not only critical for development and tissue homeostasis, but also for cancer progression, where acquired resistance to apoptosis contributes to the progression of the disease (Favaloro et al., 2012; Kelly and Strasser, 2011). The apoptotic pathways remain latent during development and are activated upon intrinsic or extrinsic apoptotic stimuli. Thus, the balanced expression of pro- and anti- apoptotic proteins has to be strictly controlled to promote cell survival or to promote cell death (Conradt, 2009; Favaloro et al., 2012; Fuchs and Steller, 2011). Protein ubiquitylation is emerging as a central mechanism in the regulation of apoptosis, controlling the amounts of pro-apoptotic and anti-apoptotic factors by promoting protein proteasomal degradation and, thereby regulating the apoptotic pathways (Bergmann, 2010; Vucic et al., 2011).

3.1. dRYBP interacts with the SCF complex to prevent apoptosis

In this thesis work, we have investigated the function of dRYBP and the SCF E3 ubiquitin ligase complex in the control of apoptosis. Our main finding is the discovery of the interaction between dRYBP and the SCF complex and their function in the inhibition of apoptosis (Table 13, Figures 15 and 18). This identifies a novel anti-apoptotic function for these proteins. Furthermore, our results suggest that dRYBP should be considered a member of the SCF E3 ligase family that promotes the ubiquitylation of many proteins (Cardozo and Pagano, 2004). As both dRYBP and the SCF complex are evolutionary conserved (Figure 7) (Bejarano et al., 2005; Bocca et al., 2001) it is also possible that the RYBP vertebrate protein is a member of the SCF complex in mammals highlighting once more the importance of *Drosophila* to discover new protein functions of the components of signaling pathways

The SCF complexes are RING-type E3-ligases that consist of the Skp1 and Cullin proteins as well as a variable F-box (Cardozo and Pagano, 2004; Jackson and Eldridge, 2002; Skowyra et al., 1997). It remains to be determined whether dRYBP is a constitutive component of the SCF complex, or if it is recruited to the SCF complex upon apoptotic and or survival stimuli. We favor the idea that it is a constitutive component as our Co-IP experiments are done in wild type embryonic nuclear protein extracts. We have demonstrated the biochemical interactions of dRYBP with SKPA and with dCUL1 (Figure 15), both core members of the SCF complex (Bocca et al., 2001; Jackson and Eldridge, 2002). Moreover, we have also found the synergistic genetic interaction between dRYBP and SKPA and dCUL1 proteins to inhibit apoptosis (Figures 16 and 18). The specification of the protein targeted for degradation depends of the F-box recruited to the complex (Skowyra et al., 1997). Results from the mass spectrometry analysis performed by dRYBP-immunoprecipitation of embryonic nuclear protein extracts (Simón, R. *et al*, manuscript in preparation) (Simón, 2013) detected very few F-box proteins interacting with dRYBP. This is perhaps due to the cellular and/or temporal context of the interaction. We chose to study the genetic interaction between dRYBP and the F-box protein Slmb as we and others have shown that lack of *slmb* function induces apoptosis (Figure 19, (Miletich and Limbourg-Bouchon, 2000). We also find a synergistic genetic interaction between *dRYBP* and *slmb* (Figures 16F and 18). These results lead us to propose that dRYBP-SCF^{Slmb} complex functions in *Drosophila* to inhibit apoptosis. This proposition may seem to contradict previous reports where SCF complexes containing different F-boxes in *Drosophila* were suggested to promote pro-apoptotic activity. For example, the SCF^{Morgue} complex promotes DIAP1 ubiquitylation and its proteasomal degradation in the eye imaginal disc (Hays et al., 2002) and the $SCF^{Nutcracker}$ complex has been shown to function in caspase activation during sperm individualization (Bader et al., 2010). We propose that this is not a contradiction but it is due to the effect of the different F-boxes that can act as a SCF member. Indeed, SCF^{bTrCP}, the

mammalian homolog of the SCF^{Slmb} in *Drosophila*, has been shown to promote Pro-Caspase 3 ubiquitylation–mediated degradation, thus protecting cells from apoptosis and functioning as a survival complex (Tan et al., 2006), similarly to the function we propose here for the dRYBP-SCF^{Slmb} complex.

We show that loss of *dRYBP* function induces cell death in a low but significant number of cells (Figures 19A and D) and that this activity is significantly increased when *skpA* is also inactivated (Figure 19J). An accumulating body of genetic and molecular evidence is supportive of a role for dRYBP/RYBP/DEDAF-conserved family in the regulation of apoptosis (Chen et al., 2009; Danen-van Oorschot et al., 2004; Gonzalez and Busturia, 2009; Novak and Phillips, 2008; Pirity et al., 2005; Stanton et al., 2007; Stanton et al., 2006; Zheng et al., 2001). Flies lacking the *dRYBP* function in *Drosophila* present either blistered or small wings (Figures 16B and 24, (Gonzalez et al., 2008)) and mice embryos lacking *Rybp* function are not able to trigger full decidualization, a process that requires apoptosis to complete (Joswig et al., 2003; Pirity et al., 2005). Additionally, inactivation of *Yaf2*, the zebrafish RYBP related protein, induces cell death in a dosage dependent morpholant manner (Stanton et al., 2006). Moreover, the dRYBP conserved protein family has been shown to interact with a range of apoptotic-related proteins, which include DD (Death Domain) and DED (Death Effector Domain) domain-containing proteins (Gonzalez and Busturia, 2009; Zheng et al., 2001), Apoptin (Danen-van Oorschot et al., 2004) and Hippi (Stanton et al., 2007). Furthermore, murine RYBP has been shown to be a regulator of the MDM2–p53 loop exhibiting tumor suppressor activity (Chen et al., 2009). Taken together, these observations provide ample evidence for the involvement of dRYBP in the control of apoptosis. Our results showing the interaction of dRYBP with the SCF^{Slmb} complex described in this thesis provides a clear starting point to begin to decipher dRYBP mechanisms of apoptosis inhibition in *Drosophila* as well as in other organisms.

3.2. dRYBP-SCF complex functions to regulates the intrinsic apoptotic pathway

Our results also show that loss of dRYBP-SCF^{Slmb} complex function has a pro-apoptotic role during wing development in *Drosophila* (Figure 16). A previous report suggested that the absence of imaginal discs in *skpA* mutant flies was due to apoptosis in imaginal cells (Murphy, 2003). Our results demonstrate that SKPA protein has a survival role during wing development (Figure 16C) acting synergistically with the dRYBP protein (Figure 16D).

In addition, we have shown that the apoptosis in both loss of *dRYBP* and *skpA* function is dependent on levels of pro-apoptotic gene *rpr* and anti-apoptotic protein DIAP1 (Figures 22, 23 and 24), indicating their role in the regulation of the intrinsic apoptotic pathway. When studying the regulation of the components of the intrinsic apoptotic pathway, we found that inactivation

of *dRYBP* does not produce any variations in *rpr* mRNA or protein levels (Figure 21D), which is probably due to the low levels of apoptosis induced in these conditions (Figures 19A, D and F). However inactivation of *skpA* promotes transcriptional activation of *rpr* expression (mRNA in S2 cells and *rpr-lacZ* in wing imaginal discs, Figures 21A-D) and also the up-regulation of Rpr protein (Figure 21E), which raised the question whether the inhibition of apoptosis mediated by dRYBP-SCF^{Slmb} complex, is at a transcriptional or at a post-translational level.

Several experimental observations made us favor the idea that the dRYBP-SCF^{Slmb}-mediated apoptosis inhibition occurs through post-translational regulation. First, it has been previously described that activation of the intrinsic apoptotic pathway up-regulates the transcription of the pro-apoptotic genes such as *rpr* (Shlevkov and Morata, 2012) and the transcription of anti-apoptotic gene *diap1* (Ryoo et al., 2002). We show that inactivation of *skpA* promotes up-regulation of *rpr* and *diap1* transcription (Figures 21 and 23B) whereas it promotes an increase in Rpr (Figure 21E) and a decrease in DIAP1 protein levels (Figure 23C). We propose that the observed activation of *rpr* and *diap1* transcription is a consequence of the activation of the intrinsic apoptotic pathway. Subsequently, Rpr protein levels increase (Figure 21E) that, in turn, repress DIAP1 protein leading to the activation of the apoptotic pathway. Second, our results show that SKPA protein directly interacts with both DIAP1 and Rpr proteins (Figure 25), further supporting a post-translational regulation through SKPA, DIAP1 and Rpr protein interactions. Third, overexpression of SKPA in HEK293 cells decreases the levels of Rpr protein (Figure 25B). It is known that protein levels of Rpr are regulated by ubiquitylation (Olson et al., 2003), thus the decrease in Rpr protein levels when co-transfected with SKPA suggests that Rpr protein is being degraded and also, that this occurs through its ubiquitylation by the SCF complex (Figure 29). Finally, the nature of the dRYBP-SCF^{Slmb} complex is to catalyze the last step of the ubiquitylation reaction of the target protein committed for proteasomal degradation (Cardozo and Pagano, 2004; Heriche et al., 2003; Inuzuka et al., 2011; Jackson and Eldridge, 2002; Tan et al., 2006) therefore, dRYBP-SCF^{Slmb} probably does not directly regulate transcription and it is most likely to function post-translationally modifying proteins for proteasomal degradation.

Consistent with the conclusion that high levels of SKPA protein decrease Rpr protein levels (Figure 25B), we also found that SKPA overexpression is capable of rescuing the effects of overexpression of Rpr (Figure 22E) and, also capable of inhibiting developmental-induced and radiation-induced apoptosis (Figure 26), further supporting its function in the inhibition of apoptosis. Curiously, high levels of dRYBP and dCUL1 also induce apoptosis (Appendix 6, (Gonzalez and Busturia, 2009)). This paradox could be explained through the interaction of dRYBP with several apoptotic-related proteins (Danen-van Oorschot et al., 2004; Gonzalez and Busturia, 2009; Stanton et al., 2007; Zheng et al., 2001). Alternatively, due to the nature of

dRYBP protein as a ubiquitin adaptor protein and of dCUL1 as a scaffold protein, perhaps high levels of these proteins recruit an excess of SCF complex components through a dominant-negative mechanism, thus interfering with SCF complex function in the inhibition of apoptosis.

Taken together, our results indicate that the inhibition of apoptosis mediated by the dRYBP-SCF^{Slmb} complex takes place through the regulation of the components of the intrinsic apoptotic pathway. Therefore, we have proposed a model (Figure 29) explaining the function of the dRYBP-SCF^{Slmb} complex in the regulation of apoptosis, whereby dRYBP functions as an ubiquitin adaptor protein facilitating the interaction and/or assembly of SCF complex members upon survival stimuli. In this model the dRYBP-SCF^{Slmb} complex may control the ubiquitylation of Rpr and its proteasomal degradation, blocking the repression of DIAP1 and, thus inhibiting cell death. However we cannot discard that the function of the dRYBP-SCF^{Slmb} could be to somehow stabilize DIAP1 protein, also known to be regulated by protein ubiquitylation (Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002), enhancing DIAP1 E3 ubiquitin ligase activity promoting Rpr ubiquitylation.

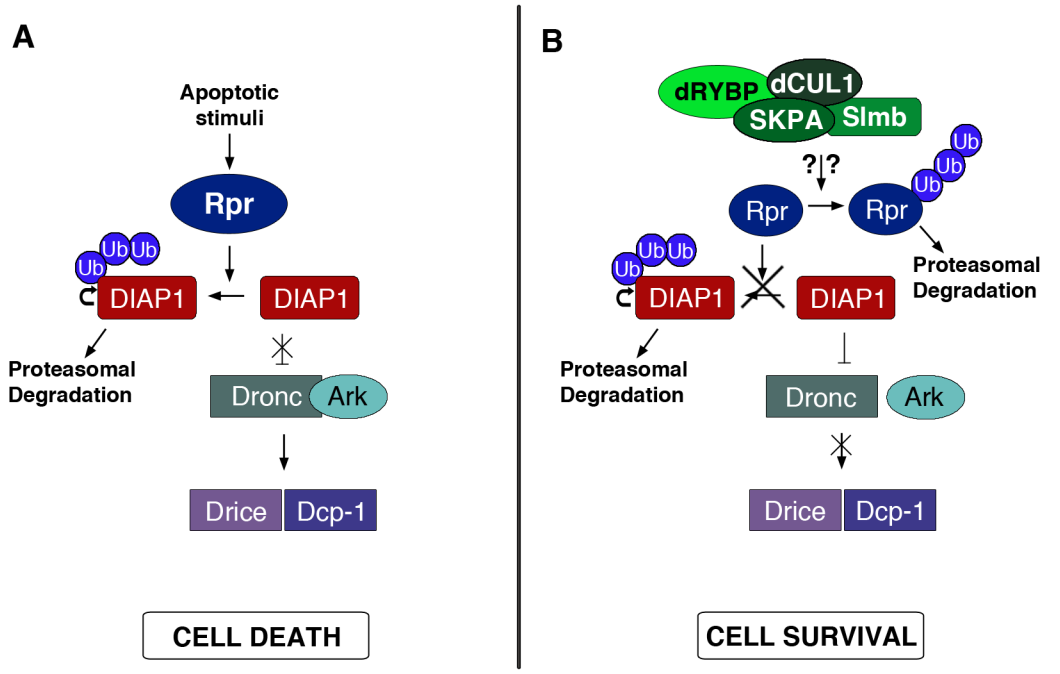


Figure 29. Proposed model for the function of dRYBP and SCF components in the inhibition of apoptosis. (**A-B**) Simplified version of the intrinsic apoptotic pathway. (**A**) Activation of the apoptotic intrinsic pathway requires the pro-apoptotic protein Rpr to repress the anti-apoptotic protein DIAP1. Rpr promotes DIAP1 autoubiquitylation, leading to proteasomal degradation {Holley, 2002 #14;Ryoo, 2002 #15;Yoo, 2002 #16} Consequently, initiator and effector caspases, Dronc and Drice and Dcp-1 respectively, are activated to execute cell death. (**B**) To ensure cell survival the intrinsic apoptotic pathway is inactivated by ubiquitylation of Rpr and subsequent proteasomal degradation. We propose that dRYBP together with the SCF complex, formed by SKPA, dCUL1 and Slmb, may promote Rpr ubiquitylation. Thus, autoubiquitylation of DIAP1 protein is inhibited and, as a result, DIAP1 is capable of inhibiting the activation of initiator and effector caspases, thereby, inhibiting cell death and promoting cell survival.

Experimental design to validate this model will help us understand the mechanisms of inhibition of apoptosis mediated by the novel dRYBP-SCF^{Slmb} complex in *Drosophila* and similar mechanisms likely to function in vertebrate development. Additionally, investigating the mechanisms of dRYBP-SCF^{Slmb} complex in *Drosophila* and in vertebrates may also have an impact on the understanding the mechanisms of inhibition of apoptosis in pathological development as well.

4. TRANSCRIPTION-DEPENDENT AND TRANSCRIPTION-INDEPENDENT FUNCTIONS OF dRYBP

During this thesis work we have discovered the function of dRYBP in epigenetic regulation of gene expression mediated by PcG and trxG proteins and also we have discovered its function in apoptosis regulation through its interaction with the SCF E3 ubiquitin ligase. Both of these functions depend on a post-translational modification of proteins that consists in ubiquitylation. How does dRYBP differentiate between these two functions? We propose that dRYBP is ubiquitously expressed therefore it will probably be interacting with PcG and trxG proteins and the SCF constitutively. However the balance between interactions may favor one or the other direction upon different stimuli. Perhaps under developmental stimuli dRYBP associates more with PcG and trxG proteins and upon survival stimuli dRYBP interacts with the SCF to inhibit apoptosis.

Our main findings are that dRYBP biochemically and genetically interacts with the PcG (SCE/dRING and dKDM2) and trxG (dBRE1) proteins (Figure 10) and also, that dRYBP modulates the levels of post-translational histone modifications (Figures 12 and 14), suggesting it is involved in the maintenance of intermediate levels of gene expression by regulating transcriptional state of genes (Figure 26). Moreover dRYBP interacts with the SCF E3 ubiquitin ligase complex to inhibit apoptosis (Figures 15 and 18) and our findings suggest that this occurs through a transcriptional-independent mechanism, particularly through the ubiquitylation and consequent proteasomal degradation of a pro-apoptotic protein (Figure 27). Interestingly, recent studies are emerging showing that PcG proteins also show transcription-independent functions such as the PSC, SCML2 and RING1B proteins. PSC protein is an E3 ubiquitin ligase that directly ubiquitylates CYCLINB for proteasomal degradation, a fundamental step for anaphase and cytokynesis progression (Mohd-Sarip et al., 2012). Likewise SCML2 (SCM homolog) is involved in the formation of the CDK2/CYCLIN/p21 repressor complex, stabilizing p21 and controlling cell cycle progression through G1 phase (Lecona et al., 2013). Additionally RFN2/RING1B (SCE/dRING homolog) protein directly binds to MDM2 and p53 proteins increasing the half-life of MDM2 protein and promoting MDM2-mediated p53 ubiquitylation

and consequently leading to p53 degradation by the proteasome, revealing its oncogenic function through a transcription-independent function (Wen et al., 2014). These findings reveal transcription-independent functions of PcG proteins supporting the transcription-independent function of the dRYBP protein through its interaction with the SCF complex.

The studies in vertebrates and in *Drosophila* demonstrate that the PcG and trxG proteins have an important role in the regulation of proteins other than histones. PcG and trxG are proteins that dynamically associate and dissociate from chromatin, for instance, during mitosis it is unlikely that these proteins bind to chromatin, thus it is essential to study not only transcriptional regulation of target genes but also transcription-independent functions to be able to understand normal and pathological development.

Conclusions

1. The dRYBP protein coexists, in *Drosophila* S2 cells, in two different forms: the dRYBP and the monoubiquitylated dRYBP (dRYBPub) proteins. Moreover, the dRYBP protein binds to ubiquitylated proteins through its N-terminal, where the NZF domain is located.

2. The dRYBP protein interacts with the H2A, H2B, H2Aub and H2Bub histones in *Drosophila* S2 cells. Additionally, *dRYBP* genetically interacts with *Sce/dRing*, *dkdm2* and *dBre1* and biochemically, in *Drosophila* wild type embryonic nuclear protein extracts (dNE), with SCE/dRING, dKDM2 and dBRE1.

3. The dRYBP protein does not biochemically interact with the PSC, PC, PH and EZ proteins and dBRE1 does not interact with the SCE/dRING, dKDM2 and EZ proteins in dNE.

4. Inactivation of *dRYBP* decreases levels of monoubiquitylated H2A (H2Aub) and monomethylated H3K4 (H3K4me).

5. dRYBP counteracts dKDM2-mediated H3K36me2 demethylation and dBRE1-mediated H2B monoubiqutylation. These dRYBP-mediated activities may therefore attenuate *dkdm2*-mediated repression and *dBRE1*-mediated activation.

6. The *dRYBP* gene interacts genetically with *skpA*, *dCul1* and *slmb*, all members of the SCF E3 ubiquitin ligase complex. Moreover, the dRYBP protein biochemically interacts with SKPA and dCUL1 proteins.

7. Inactivation of *dRYBP, skpA, dCul1* and *slmb* induces apoptosis in the wing imaginal disc and the inactivation of *skpA*- and *dRYBP*-induced apoptosis is dependent on the expression levels of the pro-apoptotic gene *rpr* and the anti-apoptotic protein DIAP1. Thus the dRYBP-SCF complex functions by inhibiting the intrinsic apoptotic pathway.

8. Inactivation of *skpA* in wing imaginal discs and in *Drosophila* S2 cells, induces the transcriptional activation of *rpr* and *diap1*, increases Rpr protein levels and decreases DIAP1 protein levels.

9. High levels of SKPA rescues the apoptotic wing phenotype induced by overexpression of Rpr. Moreover, in human HEK293 cells, SKPA protein biochemically interacts with Rpr and DIAP1 proteins and SKPA overexpression decreases Rpr protein levels, suggesting that the dRYBP-SCF complex post-translationally regulates Rpr protein levels.

10. High levels of SKPA inhibit both the apoptosis that occurs during normal leg development and X-ray-induced apoptosis.

REFERENCES

Akam, M., Averof, M., Castelli-Gair, J., Dawes, R., Falciani, F., and Ferrier, D. (1994). The evolving role of Hox genes in arthropods. Development, 209-215.

Alam, S.L., Sun, J., Payne, M., Welch, B.D., Blake, B.K., Davis, D.R., Meyer, H.H., Emr, S.D., and Sundquist, W.I. (2004). Ubiquitin interactions of NZF zinc fingers. Embo J *23*, 1411-1421.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350-355.

Aparicio, R., Neyen, C., Lemaitre, B., and Busturia, A. (2013). dRYBP contributes to the negative regulation of the Drosophila Imd pathway. PLoS One *8*, e62052.

Arrigoni, R., Alam, S.L., Wamstad, J.A., Bardwell, V.J., Sundquist, W.I., and Schreiber-Agus, N. (2006). The Polycomb-associated protein Rybp is a ubiquitin binding protein. FEBS Lett *580*, 6233-6241.

Ashburner, M. (1989). Drosophila. A laboratory Handbook.

Bader, M., Arama, E., and Steller, H. (2010). A novel F-box protein is required for caspase activation during cellular remodeling in Drosophila. Development *137*, 1679-1688.

Bejarano, F., and Busturia, A. (2004). Function of the Trithorax-like gene during Drosophila development. Dev Biol *268*, 327-341.

Bejarano, F., Gonzalez, I., Vidal, M., and Busturia, A. (2005). The Drosophila RYBP gene functions as a Polycomb-dependent transcriptional repressor. Mech Dev *122*, 1118-1129.

Bergmann, A. (2010). The role of ubiquitylation for the control of cell death in Drosophila. Cell Death Differ *17*, 61-67.

Bocca, S.N., Muzzopappa, M., Silberstein, S., and Wappner, P. (2001). Occurrence of a putative SCF ubiquitin ligase complex in Drosophila. Biochem Biophys Res Commun *286*, 357-364.

Brand, A.H., Manoukian, A.S., and Perrimon, N. (1994). Ectopic expression in Drosophila. Methods Cell Biol *44*, 635-654.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Bray, S., Musisi, H., and Bienz, M. (2005). Bre1 is required for Notch signaling and histone modification. Dev Cell *8*, 279-286.

Breen, T.R., and Duncan, I.M. (1986). Maternal expression of genes that regulate the bithorax complex of Drosophila melanogaster. Dev Biol *118*, 442-456.

Buchwald, G., van der Stoop, P., Weichenrieder, O., Perrakis, A., van Lohuizen, M., and Sixma, T.K. (2006). Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. Embo J *25*, 2465-2474.

Busturia, A., Lloyd, A., Bejarano, F., Zavortink, M., Xin, H., and Sakonju, S. (2001). The MCP silencer of the Drosophila Abd-B gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. Development *128*, 2163-2173.

Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell *20*, 845-854.

Cardozo, T., and Pagano, M. (2004). The SCF ubiquitin ligase: insights into a molecular machine. Nat Rev Mol Cell Biol *5*, 739-751.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576-1583.

Cheadle, C., Fan, J., Cho-Chung, Y.S., Werner, T., Ray, J., Do, L., Gorospe, M., and Becker, K.G. (2005). Control of gene expression during T cell activation: alternate regulation of mRNA transcription and mRNA stability. BMC Genomics *6*, 75.

Chen, D., Zhang, J., Li, M., Rayburn, E.R., Wang, H., and Zhang, R. (2009). RYBP

stabilizes p53 by modulating MDM2. EMBO Rep *10*, 166-172.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell *33*, 275-286.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J Biol Chem *257*, 2537-2542.

Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. Proc Natl Acad Sci U S A *77*, 1365-1368.

Conradt, B. (2009). Genetic control of programmed cell death during animal development. Annu Rev Genet *43*, 493-523.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185-196.

Danen-van Oorschot, A.A., Voskamp, P., Seelen, M.C., van Miltenburg, M.H., Bolk, M.W., Tait, S.W., Boesen-de Cock, J.G., Rohn, J.L., Borst, J., and Noteborn, M.H. (2004). Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. Cell Death Differ *11*, 564-573.

Davie, J.R., and Murphy, L.C. (1990). Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription. Biochemistry *29*, 4752-4757.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., *et al*. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell *7*, 663-676.

Dichtel-Danjoy, M.L., Ma, D., Dourlen, P., Chatelain, G., Napoletano, F., Robin, M., Corbet, M., Levet, C., Hafsi, H., Hainaut, P., *et al*. (2013). Drosophila p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation. Cell Death Differ *20*, 108-116.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., *et al*. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151-156.

Duncan, I. (1987). The bithorax complex. Annu Rev Genet *21*, 285-319.

Endoh, M., Endo, T.A., Endoh, T., Isono, K., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W.A., *et al*. (2012). Histone H2A mono-ubiquitination is a crucial step to mediate PRC1-dependent repression of developmental genes to maintain ES cell identity. PLoS Genet *8*, e1002774.

Estella, C., McKay, D.J., and Mann, R.S. (2008). Molecular integration of wingless, decapentaplegic, and autoregulatory inputs into Distalless during Drosophila leg development. Dev Cell *14*, 86-96.

Favaloro, B., Allocati, N., Graziano, V., Di Ilio, C., and De Laurenzi, V. (2012). Role of apoptosis in disease. Aging (Albany NY) *4*, 330-349.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Ferrandon, D. (2007). Ubiquitin-proteasome: pallbearer carries the deceased to the grave. Immunity *27*, 541-544.

Ferres-Marco, D., Gutierrez-Garcia, I., Vallejo, D.M., Bolivar, J., Gutierrez-Avino, F.J., and Dominguez, M. (2006). Epigenetic

silencers and Notch collaborate to promote malignant tumours by Rb silencing. Nature *439*, 430-436.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev *17*, 1870-1881.

Fuchs, Y., and Steller, H. (2011). Programmed cell death in animal development and disease. Cell *147*, 742-758.

Gaarenstroom, T., and Hill, C.S. (2014). TGF-beta signaling to chromatin: How Smads regulate transcription during self-renewal and differentiation. Semin Cell Dev Biol.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell *45*, 344-356.

Gildea, J.J., Lopez, R., and Shearn, A. (2000). A screen for new trithorax group genes identified little imaginal discs, the Drosophila melanogaster homologue of human retinoblastoma binding protein 2. Genetics *156*, 645-663.

Goldknopf, I.L., Taylor, C.W., Baum, R.M., Yeoman, L.C., Olson, M.O., Prestayko, A.W., and Busch, H. (1975). Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein. J Biol Chem *250*, 7182-7187.

Gonzalez, I., Aparicio, R., and Busturia, A. (2008). Functional characterization of the dRYBP gene in Drosophila. Genetics *179*, 1373-1388.

Gonzalez, I., and Busturia, A. (2009). High levels of dRYBP induce apoptosis in Drosophila imaginal cells through the activation of reaper and the requirement of trithorax, dredd and dFADD. Cell Res *19*, 747-757.

Gorfinkiel, N., Fanti, L., Melgar, T., Garcia, E., Pimpinelli, S., Guerrero, I., and Vidal, M. (2004). The Drosophila Polycomb group gene Sex combs extra encodes the ortholog of mammalian Ring1 proteins. Mech Dev *121*, 449-462.

Gutierrez, L., Oktaba, K., Scheuermann, J.C., Gambetta, M.C., Ly-Hartig, N., and Muller, J. (2012). The role of the histone H2A ubiquitinase Sce in Polycomb repression. Development *139*, 117-127.

Hay, B.A., Wassarman, D.A., and Rubin, G.M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell *83*, 1253-1262.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. Development *120*, 2121-2129.

Hays, R., Wickline, L., and Cagan, R. (2002). Morgue mediates apoptosis in the Drosophila melanogaster retina by promoting degradation of DIAP1. Nat Cell Biol *4*, 425-431.

Henry, K.W., Wyce, A., Lo, W.S., Duggan, L.J., Emre, N.C., Kao, C.F., Pillus, L., Shilatifard, A., Osley, M.A., and Berger, S.L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev *17*, 2648-2663.

Heriche, J.K., Ang, D., Bier, E., and O'Farrell, P.H. (2003). Involvement of an SCFSlmb complex in timely elimination of E2F upon initiation of DNA replication in Drosophila. BMC Genet *4*, 9.

Hershko, A., Ciechanover, A., and Rose, I.A. (1979). Resolution of the ATP-dependent proteolytic system from reticulocytes: a component that interacts with ATP. Proc Natl Acad Sci U S A *76*, 3107-3110.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem *258*, 8206-8214.

Herz, H.M., Garruss, A., and Shilatifard, A. (2013). SET for life: biochemical activities

and biological functions of SET domain-containing proteins. Trends Biochem Sci *38*, 621-639.

Hicke, L., Schubert, H.L., and Hill, C.P. (2005). Ubiquitin-binding domains. Nat Rev Mol Cell Biol *6*, 610-621.

Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkanen, R., Wagner, S., Kowanetz, K., Breitling, R., Mann, M., Stenmark, H., *et al*. (2006). Regulation of ubiquitin-binding proteins by monoubiquitination. Nat Cell Biol *8*, 163-169.

Hofmann, K., and Bucher, P. (1996). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. Trends Biochem Sci *21*, 172-173.

Holley, C.L., Olson, M.R., Colon-Ramos, D.A., and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. Nat Cell Biol *4*, 439-444.

Hubner, M.R., Eckersley-Maslin, M.A., and Spector, D.L. (2013). Chromatin organization and transcriptional regulation. Curr Opin Genet Dev *23*, 89-95.

Hughes, J.R., Meireles, A.M., Fisher, K.H., Garcia, A., Antrobus, P.R., Wainman, A., Zitzmann, N., Deane, C., Ohkura, H., and Wakefield, J.G. (2008). A microtubule interactome: complexes with roles in cell cycle and mitosis. PLoS Biol *6*, e98.

Huynh, K.D., Fischle, W., Verdin, E., and Bardwell, V.J. (2000). BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev *14*, 1810-1823.

Hwang, W.W., Venkatasubrahmanyam, S., Ianculescu, A.G., Tong, A., Boone, C., and Madhani, H.D. (2003). A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. Mol Cell *11*, 261-266.

Inuzuka, H., Shaik, S., Onoyama, I., Gao, D., Tseng, A., Maser, R.S., Zhai, B., Wan, L., Gutierrez, A., Lau, A.W., *et al*. (2011). SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. Nature *471*, 104-109.

Jackle, H., Hoch, M., Pankratz, M.J., Gerwin, N., Sauer, F., and Bronner, G. (1992). Transcriptional control by Drosophila gap genes. J Cell Sci Suppl *16*, 39-51.

Jackson, P.K., and Eldridge, A.G. (2002). The SCF ubiquitin ligase: an extended look. Mol Cell *9*, 923-925.

Jiang, C., Lamblin, A.F., Steller, H., and Thummel, C.S. (2000). A steroid-triggered transcriptional hierarchy controls salivary gland cell death during Drosophila metamorphosis. Mol Cell *5*, 445-455.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature *391*, 493-496.

Joswig, A., Gabriel, H.D., Kibschull, M., and Winterhager, E. (2003). Apoptosis in uterine epithelium and decidua in response to implantation: evidence for two different pathways. Reprod Biol Endocrinol *1*, 44.

Kanayama, A., Seth, R.B., Sun, L., Ea, C.K., Hong, M., Shaito, A., Chiu, Y.H., Deng, L., and Chen, Z.J. (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell *15*, 535-548.

Kao, C.F., Hillyer, C., Tsukuda, T., Henry, K., Berger, S., and Osley, M.A. (2004). Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. Genes Dev *18*, 184-195.

Kaufman, T.C., Seeger, M.A., and Olsen, G. (1990). Molecular and genetic organization of the antennapedia gene complex of Drosophila melanogaster. Adv Genet *27*, 309-362.

Kelly, G.L., and Strasser, A. (2011). The essential role of evasion from cell death in cancer. Adv Cancer Res *111*, 39-96.

Kennison, J.A., and Tamkun, J.W. (1988). Dosage-dependent modifiers of polycomb and antennapedia mutations in Drosophila. Proc Natl Acad Sci U S A *85*, 8136-8140.

Kerridge, S.a.T.-C., M. (1988). Appendage morphogenesis in Drosophila: a developmental study of the rotund (rn) gene Roux's Arch Dev Biol *197*, 19-26.

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715-727.

Klymenko, T., Papp, B., Fischle, W., Kocher, T., Schelder, M., Fritsch, C., Wild, B., Wilm, M., and Muller, J. (2006). A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. Genes Dev *20*, 1110-1122.

Krumlauf, R. (1994). Hox genes in vertebrate development. Cell *78*, 191-201.

Kulathu, Y., and Komander, D. (2012). Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat Rev Mol Cell Biol *13*, 508-523.

Lagarou, A., Mohd-Sarip, A., Moshkin, Y.M., Chalkley, G.E., Bezstarosti, K., Demmers, J.A., and Verrijzer, C.P. (2008). dKDM2 couples histone H2A ubiquitylation to histone H3 demethylation during Polycomb group silencing. Genes Dev *22*, 2799-2810.

Lau, A.W., Fukushima, H., and Wei, W. (2012). The Fbw7 and betaTRCP E3 ubiquitin ligases and their roles in tumorigenesis. Front Biosci *17*, 2197-2212.

Lawrence, P. (1992). The Making of a fly. The genetics of Animal Design.

Lecona, E., Rojas, L.A., Bonasio, R., Johnston, A., Fernandez-Capetillo, O., and Reinberg, D. (2013). Polycomb protein SCML2 regulates the cell cycle by binding and modulating CDK/CYCLIN/p21 complexes. PLoS Biol *11*, e1001737.

Lewis, E.B. (1978). A gene complex controlling segmentation in Drosophila. Nature *276*, 565-570.

MacKenzie, S.H., and Clark, A.C. (2012). Death by caspase dimerization. Adv Exp Med Biol *747*, 55-73.

Manjon, C., Sanchez-Herrero, E., and Suzanne, M. (2007). Sharp boundaries of Dpp signalling trigger local cell death required for Drosophila leg morphogenesis. Nat Cell Biol *9*, 57-63.

Mann, M., and Jensen, O.N. (2003). Proteomic analysis of post-translational modifications. Nat Biotechnol *21*, 255-261.

Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A.M., and Martinez-Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes Dev *12*, 557-570.

Martinez, A.M., Colomb, S., Dejardin, J., Bantignies, F., and Cavalli, G. (2006). Polycomb group-dependent Cyclin A repression in Drosophila. Genes Dev *20*, 501-513.

Martinez-Arias, A., and Lawrence, P.A. (1985). Parasegments and compartments in the Drosophila embryo. Nature *313*, 639-642.

Miletich, I., and Limbourg-Bouchon, B. (2000). Drosophila null slimb clones transiently deregulate Hedgehog-independent transcription of wingless in all limb discs, and induce decapentaplegic transcription linked to imaginal disc regeneration. Mech Dev *93*, 15-26.

Mohan, M., Herz, H.M., Smith, E.R., Zhang, Y., Jackson, J., Washburn, M.P., Florens, L., Eissenberg, J.C., and Shilatifard, A. (2011). The COMPASS family of H3K4 methylases in Drosophila. Mol Cell Biol *31*, 4310-4318.

Mohd-Sarip, A., Lagarou, A., Doyen, C.M., van der Knaap, J.A., Aslan, U., Bezstarosti, K., Yassin, Y., Brock, H.W., Demmers, J.A., and Verrijzer, C.P. (2012). Transcription-independent function of Polycomb group protein PSC in cell cycle control. Science *336*, 744-747.

Moreno, E., Yan, M., and Basler, K. (2002). Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog

of the TNF superfamily. Curr Biol *12*, 1263-1268.

Morey, L., Aloia, L., Cozzuto, L., Benitah, S.A., and Di Croce, L. (2013). RYBP and Cbx7 define specific biological functions of polycomb complexes in mouse embryonic stem cells. Cell Rep *3*, 60-69.

Mulholland, N.M., King, I.F., and Kingston, R.E. (2003). Regulation of Polycomb group complexes by the sequence-specific DNA binding proteins Zeste and GAGA. Genes Dev *17*, 2741-2746.

Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197-208.

Muller, J., and Kassis, J.A. (2006). Polycomb response elements and targeting of Polycomb group proteins in Drosophila. Curr Opin Genet Dev *16*, 476-484.

Muller, J., and Verrijzer, P. (2009). Biochemical mechanisms of gene regulation by polycomb group protein complexes. Curr Opin Genet Dev *19*, 150-158.

Murphy, T.D. (2003). Drosophila skpA, a component of SCF ubiquitin ligases, regulates centrosome duplication independently of cyclin E accumulation. J Cell Sci *116*, 2321-2332.

Nakamura, K., Kato, A., Kobayashi, J., Yanagihara, H., Sakamoto, S., Oliveira, D.V., Shimada, M., Tauchi, H., Suzuki, H., Tashiro, S., *et al.* (2011). Regulation of homologous recombination by RNF20-dependent H2B ubiquitination. Mol Cell *41*, 515-528.

Nakanishi, S., Lee, J.S., Gardner, K.E., Gardner, J.M., Takahashi, Y.H., Chandrasekharan, M.B., Sun, Z.W., Osley, M.A., Strahl, B.D., Jaspersen, S.L., *et al.* (2009). Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. J Cell Biol *186*, 371-377.

Novak, R.L., and Phillips, A.C. (2008). Adenoviral-mediated Rybp expression promotes tumor cell-specific apoptosis. Cancer Gene Ther *15*, 713-722.

Oktaba, K., Gutierrez, L., Gagneur, J., Girardot, C., Sengupta, A.K., Furlong, E.E., and Muller, J. (2008). Dynamic regulation by polycomb group protein complexes controls pattern formation and the cell cycle in Drosophila. Dev Cell *15*, 877-889.

Olson, M.R., Holley, C.L., Yoo, S.J., Huh, J.R., Hay, B.A., and Kornbluth, S. (2003). Reaper is regulated by IAP-mediated ubiquitination. J Biol Chem *278*, 4028-4034.

Osley, M.A. (2004). H2B ubiquitylation: the end is in sight. Biochim Biophys Acta *1677*, 74-78.

Pandey, U.B., and Nichols, C.D. (2011). Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. Pharmacol Rev *63*, 411-436.

Pirity, M.K., Locker, J., and Schreiber-Agus, N. (2005). Rybp/DEDAF is required for early postimplantation and for central nervous system development. Mol Cell Biol *25*, 7193-7202.

Probst, A.V., Dunleavy, E., and Almouzni, G. (2009). Epigenetic inheritance during the cell cycle. Nat Rev Mol Cell Biol *10*, 192-206.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., and Bier, E. (2001). A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res *11*, 1114-1125.

Robzyk, K., Recht, J., and Osley, M.A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. Science *287*, 501-504.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348-353.

Ryoo, H.D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. (2002). Regulation of Drosophila IAP1 degradation and apoptosis by reaper and ubcD1. Nat Cell Biol *4*, 432-438.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual.

Sanchez-Herrero, E., Casanova, J., Kerridge, S., and Morata, G. (1985a). Anatomy and function of the bithorax complex of Drosophila. Cold Spring Harb Symp Quant Biol *50*, 165-172.

Sanchez-Herrero, E., Vernos, I., Marco, R., and Morata, G. (1985b). Genetic organization of Drosophila bithorax complex. Nature *313*, 108-113.

Sandu, C., Ryoo, H.D., and Steller, H. (2010). Drosophila IAP antagonists form multimeric complexes to promote cell death. J Cell Biol *190*, 1039-1052.

Schlisio, S., Halperin, T., Vidal, M., and Nevins, J.R. (2002). Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. Embo J *21*, 5775-5786.

Schuettengruber, B., and Cavalli, G. (2010). The DUBle life of polycomb complexes. Dev Cell *18*, 878-880.

Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet *8*, 9-22.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell *98*, 37-46.

Shilatifard, A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem *75*, 243-269.

Shlevkov, E., and Morata, G. (2012). A dp53/JNK-dependant feedback amplification loop is essential for the apoptotic response to stress in Drosophila. Cell Death Differ *19*, 451-460.

Silverman, J.S., Skaar, J.R., and Pagano, M. (2012). SCF ubiquitin ligases in the maintenance of genome stability. Trends Biochem Sci *37*, 66-73.

Simon, J., Chiang, A., and Bender, W. (1992). Ten different Polycomb group genes are required for spatial control of the abdA and AbdB homeotic products. Development *114*, 493-505.

Simón, R. (2013). Función de las proteínas Polycomb/Trithorax y Dp53 en la regulación de la expresión génica (Universidad Autónoma de Madrid).

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209-219.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S., and Rubin, G.M. (1999). The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. Genetics *153*, 135-177.

Stanton, S.E., Blanck, J.K., Locker, J., and Schreiber-Agus, N. (2007). Rybp interacts with Hippi and enhances Hippi-mediated apoptosis. Apoptosis *12*, 2197-2206.

Stanton, S.E., McReynolds, L.J., Evans, T., and Schreiber-Agus, N. (2006). Yaf2 inhibits caspase 8-mediated apoptosis and regulates cell survival during zebrafish embryogenesis. J Biol Chem *281*, 28782-28793.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science *267*, 1445-1449.

Sun, Z.W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104-108.

Sundqvist, A., Bengoechea-Alonso, M.T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J.W., and Ericsson, J. (2005). Control of lipid metabolism by phosphorylation-

dependent degradation of the SREBP family of transcription factors by SCF(Fbw7). Cell Metab *1*, 379-391.

Tan, M., Gallegos, J.R., Gu, Q., Huang, Y., Li, J., Jin, Y., Lu, H., and Sun, Y. (2006). SAG/ROC-SCF beta-TrCP E3 ubiquitin ligase promotes pro-caspase-3 degradation as a mechanism of apoptosis protection. Neoplasia *8*, 1042-1054.

Tanny, J.C., Erdjument-Bromage, H., Tempst, P., and Allis, C.D. (2007). Ubiquitylation of histone H2B controls RNA polymerase II transcription elongation independently of histone H3 methylation. Genes Dev *21*, 835-847.

Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., *et al*. (2012). RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell *148*, 664-678.

Thompson, S.J., Loftus, L.T., Ashley, M.D., and Meller, R. (2008). Ubiquitin-proteasome system as a modulator of cell fate. Curr Opin Pharmacol *8*, 90-95.

Thornton, J.L., Westfield, G.H., Takahashi, Y.H., Cook, M., Gao, X., Woodfin, A.R., Lee, J.S., Morgan, M.A., Jackson, J., Smith, E.R., *et al*. (2014). Context dependency of Set1/COMPASS-mediated histone H3 Lys4 trimethylation. Genes Dev *28*, 115-120.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

Van der Knaap, J.A., Kozhevnikova, E., Langenberg, K., Moshkin, Y.M., and Verrijzer, C.P. (2010). Biosynthetic enzyme GMP synthetase cooperates with ubiquitin-specific protease 7 in transcriptional regulation of ecdysteroid target genes. Mol Cell Biol *30*, 736-744.

Vidal, M., and Cagan, R.L. (2006). Drosophila models for cancer research. Curr Opin Genet Dev *16*, 10-16.

Vucic, D., Dixit, V.M., and Wertz, I.E. (2011). Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol *12*, 439-452.

Walter, D., Matter, A., and Fahrenkrog, B. (2010). Bre1p-mediated histone H2B ubiquitylation regulates apoptosis in Saccharomyces cerevisiae. J Cell Sci *123*, 1931-1939.

Wang, B., Alam, S.L., Meyer, H.H., Payne, M., Stemmler, T.L., Davis, D.R., and Sundquist, W.I. (2003). Structure and ubiquitin interactions of the conserved zinc finger domain of Npl4. J Biol Chem *278*, 20225-20234.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873-878.

Wen, W., Peng, C., Kim, M.O., Ho Jeong, C., Zhu, F., Yao, K., Zykova, T., Ma, W., Carper, A., Langfald, A., *et al*. (2014). Knockdown of RNF2 induces apoptosis by regulating MDM2 and p53 stability. Oncogene *33*, 421-428.

Wichmann, A., Jaklevic, B., and Su, T.T. (2006). Ionizing radiation induces caspase-dependent but Chk2- and p53-independent cell death in Drosophila melanogaster. Proc Natl Acad Sci U S A *103*, 9952-9957.

Wing, J.P., Schwartz, L.M., and Nambu, J.R. (2001). The RHG motifs of Drosophila Reaper and Grim are important for their distinct cell death-inducing abilities. Mech Dev *102*, 193-203.

Wright, D.E., Wang, C.Y., and Kao, C.F. (2011). Flickin' the ubiquitin switch: the role of H2B ubiquitylation in development. Epigenetics *6*, 1165-1175.

Xu, J., Matsuzaki, K., McKeehan, K., Wang, F., Kan, M., and

McKeehan, W.L. (1994). Genomic structure and cloned cDNAs predict that four variants in the kinase domain of serine/threonine kinase receptors arise by alternative splicing and poly(A) addition. Proc Natl Acad Sci U S A *91*, 7957-7961.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., and Hay, B.A. (2002). Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat Cell Biol *4*, 416-424.

Young, P., Deveraux, Q., Beal, R.E., Pickart, C.M., and Rechsteiner, M. (1998). Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a. J Biol Chem *273*, 5461-5467.

Zhang, W., and Sidhu, S.S. (2014). Development of inhibitors in the ubiquitination cascade. FEBS Lett *588*, 356-367.

Zhang, Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. Genes Dev *17*, 2733-2740.

Zheng, L., Schickling, O., Peter, M.E., and Lenardo, M.J. (2001). The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. J Biol Chem *276*, 31945-31952.

Zinngrebe, J., Montinaro, A., Peltzer, N., and Walczak, H. (2014). Ubiquitin in the immune system. EMBO Rep *15*, 322.

RESUMEN EN CASTELLANO

1. INTRODUCCIÓN

La regulación de la expresión génica es fundamental para el desarrollo normal y patológico de los organismos ya que su desregulación puede generar defectos morfológicos y enfermedades tales como el cáncer o enfermedades neurodegenerativas. La mosca *Drosophila melanogaster* es un sistema muy útil para el estudio de la regulación de la expresión génica, ya que posee unas características biológicas idóneas como son su pequeño tamaño, su ciclo de vida corto y su elevada fertilidad. Muy importantemente, las vías de señalización que regulan el desarrollo de esta mosca están muy conservadas en la evolución. Además, el uso de *Drosophila* ha promovido la generación de herramientas experimentales tanto genéticas como moleculares que no están disponibles en ningún otro sistema modelo, haciendo de este organismo un modelo biológico de elección.

Existen numerosos mecanismos, que tienen lugar a diferentes niveles, implicados en la correcta regulación de la expresión génica. La vía de la ubiquitinación está emergiendo como un proceso fundamental para la regulación de la expresión génica. El proceso de ubiquitinación está filogenéticamente conservado y consiste en la adición covalente de una o más moléculas de ubiquitina a la lisina de una proteína diana. Dependiendo de las moléculas de ubiquitina unidas y de cómo se unen unas a otras, las consecuencias fisiológicas en la proteína diana pueden ser variables. Por ejemplo, la monoubiquitinación de histonas está implicada en la regulación de la expresión génica, mientras que la ubiquitinación a través de la -K48 es responsable de la degradación vía proteasoma de la proteína diana, un proceso esencial para mantener los niveles de proteínas en distintas vías como la apoptosis. En esta tesis se han estudiado los mecanismos implicados en la regulación epigenética de la expresión génica y en la apoptosis que controlan el desarrollo de *Drosophila*.

La regulación epigenética de la expresión génica esta mediada por las proteínas Polycomb (PcG) y trithorax (trxG). Estas proteínas se encargan de mantener los estados transcripcionales reprimidos y activados respectivamente durante el desarrollo y la vida adulta. PcG y trxG son proteínas nucleares asociadas a cromatina que se expresan de forma ubicua y que regulan el mantenimiento de la expresión de una gran variedad de genes implicados en múltiples procesos biológicos como proliferación, mantenimiento de la pluripotencialidad de células madre y tumorogénesis. Existen al menos 40 proteínas PcG y trxG descritas por el momento. Los mecanismos de acción de estas proteínas consisten en tres pasos. 1) Unión de proteínas PcG o trxG con dominios de unión a ADN, como PHO y Trl, a secuencias específicas denominadas Polycomb Response Elements (PREs) y Trithorax Response Elements (TREs) en los genes diana. 2) Reclutamiento de proteínas PcG y trxG al ADN formando complejos multiméricos. 3) Modificación post-traduccional de histonas, como metilación, acetilación y ubiquitinación, generando distintos estados de compactación

de la cromatina, resultando en distintos niveles de transcripción. Además existe un tercer grupo de genes que codifican para proteínas capaces de interaccionar tanto con proteínas PcG y trxG denominados ETP (Enhancers of Trithorax and Polycomb). Entre los ETPs se encuentra la proteína dRYBP, objeto de estudio de este trabajo de tesis.

El gen *dRYBP* (Ring1B and Yin yang1 Binding Protein) está filogenéticamente conservado y codifica para una proteína de 17kDa que se expresa de forma ubicua a lo largo del desarrollo. dRYBP contiene en su N-terminal un dominio de unión a ubiquitina (UBD) del tipo NZF (Nucleoporin Zinc Finger). La falta de función de *dRYBP* produce diferentes fenotipos que son variables en penetrancia y expresividad, como reducción del tamaño de los órganos y esterilidad en hembras, sugiriendo el papel de dRYBP en multitud de procesos biológicos. Además, también se sabe que altos niveles de la proteína dRYBP inducen apoptosis en la mosca y que su homólogo en vertebrados, RYBP, induce apoptosis en células transformadas. Por otro lado, tanto en *Drosophila* como en vertebrados, se ha descrito la interacción de dRYBP/RYBP con diferentes proteínas relacionadas con apoptosis. Sin embargo, los mecanismos moleculares a través de los cuales dRYBP/RYBP regulan la apoptosis aún se desconocen.

La apoptosis es un proceso que tiene lugar durante el desarrollo para asegurar la correcta morfogénesis del individuo y durante de la vida adulta para eliminar células dañadas. Los mecanismos que controlan la apoptosis se encuentran conservados en la evolución y consisten, en último término, en la activación de una familia de proteasas, las caspasas, a través de señales intrínsecas o extrínsecas. En *Drosophila,* la vía intrínseca consiste en la activación de las proteínas pro-apoptóticas Reaper, Hid y Grim (RHG) (Wing et al., 2001), que inhiben a DIAP1 (*Drosophila* Inhibitor of Apoptosis Protein 1) (Hay et al., 1995). Consecuentemente, DIAP1 no puede inhibir a la caspasa iniciadora Dronc, que entonces es capaz de activar a las caspasas efectoras Drice y Dcp-1 produciendo la muerte celular (Fuchs and Steller, 2011; MacKenzie and Clark, 2012; Steller, 1995). Tanto las proteínas RHG como DIAP1 son capaz de ubiquitinarse para ser degradadas por el proteasoma. Por lo tanto, los niveles de RHG así como los de DIAP1 deben ser estrictamente regulados para mantener el balance entre muerte y supervivencia celular, asegurando así el correcto desarrollo del organismo.

2. OBJETIVOS

Los objetivos de este trabajo de tesis fueron estudiar la funcionalidad de la proteína dRYBP en dos procesos en los que la ubiquitinación juega un papel esencial: la regulación

epigenética de la expresión génica mediada por las proteínas PcG y trxG y la regulación de la apoptosis utilizando *Drosophila* como sistema modelo.

Los objetivos específicos de este trabajo de tesis fueron:

1. Análisis de la función molecular de la proteína dRYBP

2. Estudio de la función de dRYBP en la regulación epigenética de la expresión génica

3. Estudio de la función de dRYBP en la regulación de la apoptosis

3. RESULTADOS

3.1. Análisis de la función molecular de la proteína dRYBP

Mediante el análisis en células S2 de *Drosophila* y su posterior detección mediante WB hemos descubierto que la proteína dRYBP coexiste en dos formas: dRYBP y dRYBP monoubiquitinada (dRYBPub). Además, hemos generado dos construcciones génicas, una, con la proteína silvestre y otra, una forma truncada en el que el dominio NZF, que contiene el UBD, se encuentra deleccionado. A través de ensayos de "pulldown" con extractos de proteínas de células S2 hemos descubierto que el dominio NZF es fundamental para la unión de dRYBP a proteínas ubiquitinadas. Además, realizando este mismo tipo de ensayos pero usando histonas, hemos descubierto que dRYBP interacciona con las histonas H2A, H2A ubiquitinada (H2Aub), H2B y H2B ubiquitinada (H2Bub), confirmando su papel en la regulación epigenética de la expresión génica.

3.2. Estudio de la función de dRYBP en la regulación epigenética de la expresión génica

Ensayos de co-inmunoprecipitación con extractos proteicos nucleares de embriones de *Drosophila* (dNE) muestran que dRYBP interacciona bioquímicamente, por un lado, con las proteínas PcG SCE/dRING y dKDM2 y, por otro lado, con la proteína trxG dBRE1. Sin embargo dRYBP no interacciona bioquímicamente con las proteínas PcG PSC, PC, PH y EZ. Además, a través de la inactivación de *dRYBP, Sce/dRing, dkdm2* y *dBre1* en *Drosophila* y el análisis fenotípico de los individuos resultantes demuestran que *dRYBP* interacciona con *Sce/dRing*, con *dkdm2* para contrarrestar la represión mediada por dKDM2 y con *dBre1* para contrarrestar la activación mediada por dBRE1. Para estudiar el papel de dRYBP en la regulación epigenética mediada por PcG y trxG inactivamos por un lado, *dRYBP, Sce/dRing, dkdm2* y *dBre1* independientemente y, por otro lado, la inactivación simultánea de *dRYBP* y *Sce/dRing, dkdm2* o *dBre1* en células S2 posteriormente analizamos mediante WB los niveles de modificaciones post-traduccionales de las histonas. Los resultados muestran que dRYBP

promueve, de forma directa o indirecta, la ubiquitinación de H2A y la metilación de la H3K4. Los resultados también indican que dRYBP es capaz de modular la demetilación de la H3K36me2 mediada por dKDM2 y de modular la ubiquitinación de la H2Bub mediada por dBRE1.

3.3. Estudio de la función de dRYBP en la regulación de la apoptosis

Realizando ensayos de co-inmunoprecipitación con dNE hemos demostrado que dRYBP interacciona bioquímicamente con las proteínas SKPA y dCUL1, componentes del complejo SCF E3 ubiquitin ligasa. Además la inactivación en el ala simultanea de *dRYBP* y *skpA, dCUL1* o *slmb* muestran que *dRYBP* interacciona genéticamente con el complejo SCF E3 ubiquitin ligasa y que este complejo es dRYBP-SCF^{Slmb} es fundamental para la supervivencia de la mosca. El análisis de la falta de función de cada uno de los componentes del complejo dRYBP-SCF^{Slmb} en el disco imaginal de ala y el posterior análisis mediante inmunohistoquímica de la expresión de caspasa-3-activada (C3), muestran que su inactivación induce expresión de C3 y, por tanto, revela el papel anti-apoptótico de este complejo. El estudio de la expresión de distintos marcadores a través de técnicas inmunohistoquímicas en el disco imaginal de ala y del análisis mediante RT-PCR cuantitativa en células S2 demuestran la regulación por parte de las proteínas dRYBP y SKPA de la vía apoptótica intrínseca. En concreto, la apoptosis inducida por la falta de función de *dRYBP* y *skpA* es dependiente de los niveles de expresión del gen pro-apoptótico *rpr* y de la proteína anti-apoptótica DIAP1. Además la falta de función de *skpA* induce expresión transcripcional de los genes *rpr* y *diap1*, aumenta los niveles de la proteína Rpr y disminuye los niveles de la proteína DIAP1. La proteína SKPA interacciona bioquímicamente con Rpr y DIAP1 en células S2. Nuestros resultados también indican que la sobre-expresión de SKPA disminuye los niveles de expresión de la proteína Rpr en células S2 y también es capaz de rescatar el fenotipo apoptótico que se produce en el ala por la sobre-expresión de Rpr. Por último, hemos mostrado que la sobre-expresión de SKPA es capaz de inhibir la apoptosis que tiene lugar durante el desarrollo y la apoptosis que se produce en respuesta a estrés, confirmando el papel anti-apoptótico del complejo dRYBP-SCF^{Slmb}.

4. DISCUSIÓN

En primer lugar, hemos estudiado la funcionalidad del dominio UBD de la proteína dRYBP. Nuestros resultados indican que este dominio es esencial para que dRYBP interaccione con proteínas ubiquitinadas, sugiriendo el posible papel de dRYBP como proteína adaptadora de ubiquitina. Estos datos concuerdan con los resultados previos en vertebrados, en los que muestran que RYBP se une a proteínas ubiquitinadas y ubiquitina a

través del NZF. Esta posible función de dRYBP como proteína adaptadora de ubiquitina podría explicar la variedad de fenotipos asociados a la falta de función de dRYBP y la diversidad de proteínas con las que interacciona.

También hemos estudiado la función de dRYBP en la regulación epigenética de la expresión génica mediada por PcG y trxG, Nuestros resultados indican que dRYBP forma un complejo represor junto con las proteínas PcG SCE/dRING y dKDM2 (complejo que hemos denominado dRRK) y un complejo activador junto con la proteína dBRE1 (complejo que hemos denominado dRB). El análisis de las interacciones genéticas y moleculares entre los componentes de estos complejo, al igual que el estudio de las modificaciones post-traduccionales de las histonas, sugieren que el complejo dRRK lleva a cabo la represión génica a través de la ubiquitinación de la H2A. Además, la interacción entre dRYBP y dKDM2 contrarresta la demetilación de la H3K36me2 mediada por dKDM2, promoviendo niveles intermedios de H3K36me2 y H3K36me, que podría generar un estado transcripcional intermedio de represión. Por otro lado, nuestros datos también indican que la interacción entre dRYBP y dBRE1 en el complejo dRB contrarresta los niveles de H2Bub mediados por dBRE1, sugiriendo la generación de estados transcripcionales intermedios de activación. Por tanto, la presencia de dRYBP podría ser fundamental para promover niveles intermedios de expresión génica y que podrían estar involucrados en promover la plasticidad epigenética de la regulación mediada por los complejos PcG y trxG durante el desarrollo normal y patológico de los individuos

Por último, el hallazgo de la interacción entre dRYBP y el complejo SCF revela una función de dRYBP independiente de la transcripción en la regulación de la apoptosis. Nuestros resultados indican que este complejo dRYBP-SCF^{Slmb} tiene un papel esencial en la inhibición de la vía apoptótica y además, sugieren un modelo en el que esta inhibición ocurre a través de la ubiquitinación de la proteína pro-apoptótica Rpr, para ser degradada por el proteasoma. La validación de este modelo y su estudio en vertebrados podría ayudar a entender mejor los mecanismos moleculares de la inhibición de la apoptosis en el desarrollo normal y de enfermedades

En resumen, el análisis genético y bioquímico en este trabajo de tesis revela el papel de dRYBP en la regulación epigenética de la expresión génica y en la regulación de la muerte celular. dRYBP controla estos procesos mediante dos mecanismos: uno, dependiente de transcripción donde dRYBP, junto con las proteínas PcG y trxG, modula los niveles de modificaciones post-traduccionales de histonas. El segundo mecanismo, independiente de la transcripción, donde dRYBP regula la apoptosis a través de su interacción con el complejo

SCF E3 ubiquitin ligasa, involucrado en la degradación por el proteasoma de proteínas. Este papel dual de dRYBP dependiente e independiente de la transcripción concuerda con estudios recientes en los que se han visto que otras proteínas del grupo PcG tienen una función independiente de las transcripción. Por ejemplo, la proteína PSC ubiquitina a la CiclinaB, permitiendo la progresión del ciclo celular (Mohd-Sarip et al., 2012). Por lo tanto, el estudio de las proteínas PcG no solo debe centrarse en regulación epigenética si no también en dianas independientes de la transcripción para comprender mejor las bases moleculares del desarrollo de los organismos.

5. CONCLUSIONES

1. La proteína dRYBP coexiste en células S2 de *Drosophila* en dos formas: dRYBP y dRYBP monoubiquitinada (dRYBPub). Además la proteína dRYBP se une a proteínas ubiquitinadas a través de su N-terminal, donde se encuentra localizado su dominio NZF.

2. La proteína dRYBP interacciona con las histonas H2A, H2B, H2Aub and H2Bub en células S2 de *Drosophila*. dRYBP interacciona genéticamente con *Sce/dRing*, *dkdm2* y *dBre1* y bioquímicamente, en extractos de proteínas nucleares de embriones de *Drosophila* (dNE), con SCE/dRING, dKDM2 y dBRE1.

3. dRYBP no interacciona bioquímicamente con las proteínas PSC, PC, PH y EZ y dBRE1 no interacciona con las proteínas SCE/dRING, dKDM2 and EZ en dNE.

4. La inactivación de *dRYBP* disminuye los niveles de H2A monoubiquitinada (H2Aub) y de H3K4 monometilada (H3K4me).

5. dRYBP contrarresta la demetilación de H3K36me2 mediada por dKDM2 y la ubiquitinación de H2B ubiquitinada (H2Bub) mediados por dBRE1. Estas actividades de dRYBP, por lo tanto, podrían atenuar la represión mediada por *dkdm2* y la activación mediada por *dBre1*.

6. El gen *dRYBP* interacciona genéticamente con *skpA*, *dCul1* y *slmb*, componentes del complejo SCF E3 ubiquitin ligasa. Además dRYBP interacciona bioquímicamente con las proteínas SKPA y dCUL1.

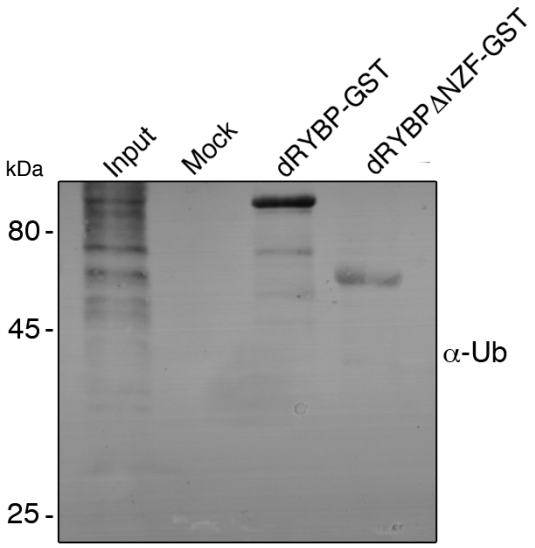
7. La inactivación de *dRYBP, skpA, dCul1* y *slmb* induce apoptosis en el disco imaginal de ala y además la apoptosis producida por la inactivación de *skpA* y *dRYBP* es dependiente de la expresión del gen pro-apoptótico *rpr* y de la proteína anti-apoptótica DIAP1. Por lo tanto, la función del complejo dRYBP-SCF es inhibir la vía apoptótica intrínseca.

8. La inactivación de *skpA* en el disco imaginal de ala y en células S2 de *Drosophila*, induce la activación transcripcional de *rpr* y *diap1*, aumenta los niveles de la proteína Rpr y disminuye los niveles de la proteína DIAP1.

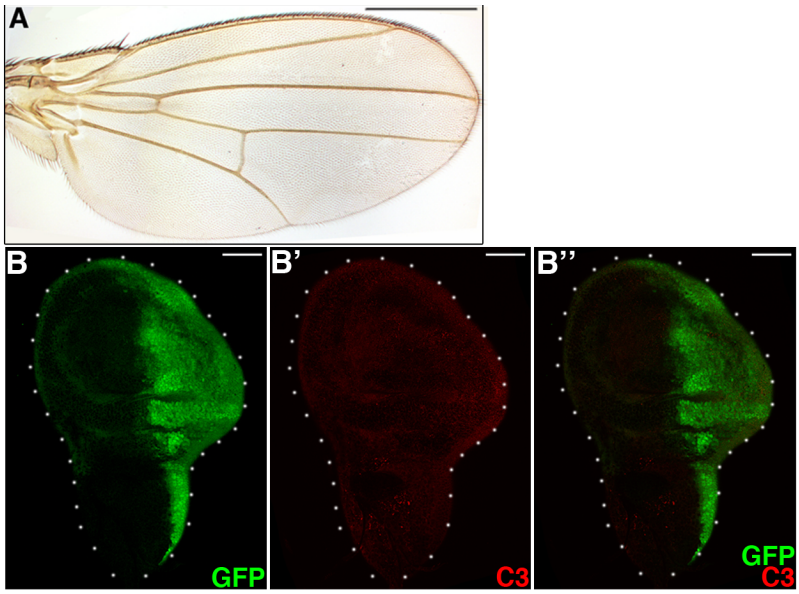
9. Altos niveles de SKPA rescatan el fenotipo apoptótico en el ala inducido por la sobre-expresión de Rpr. Además, en células humanas HEK293 la proteína SKPA interacciona bioquímicamente con las proteínas Rpr y DIAP1 y la sobre-expresión de SKPA reduce los niveles de la proteína Rpr, sugiriendo que el complejo dRYBP-SCF modifica post-traduccionalmente a la proteína Rpr.

10. Altos niveles de SKPA inhiben la apoptosis que tiene lugar durante el desarrollo de la las patas y la apoptosis que tiene lugar en respuesta a rayos-X.

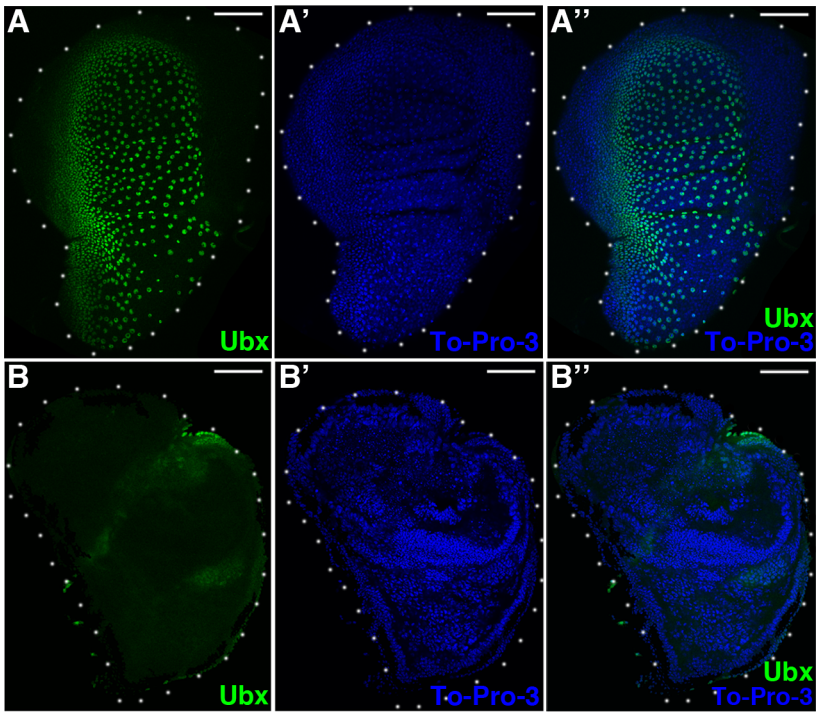
Appendix



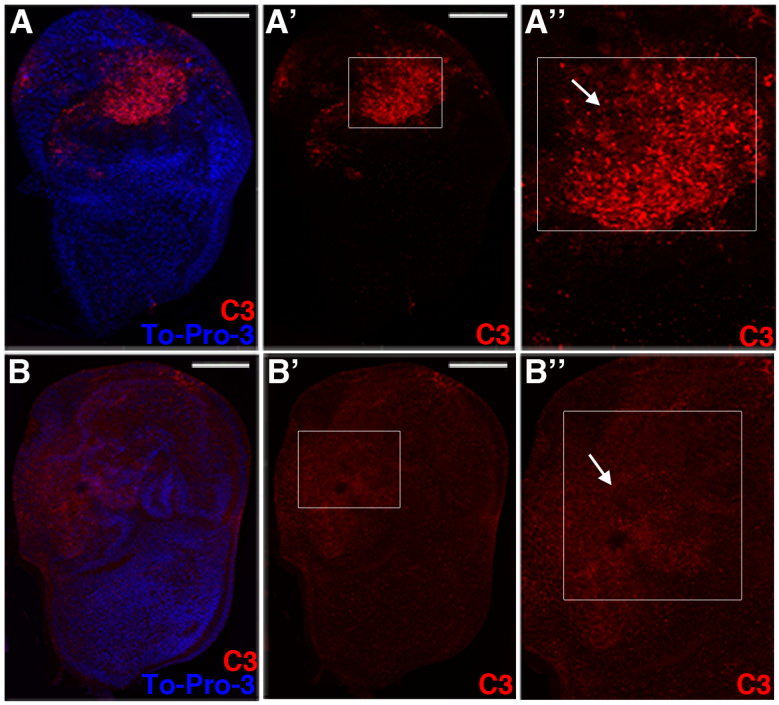
APPENDIX 1. dRYBP pulldown assay in wild type S2 cell extracts. GST-pulldown assay using wild type S2 cell extracts (Input) incubated with GST beads (Mock) or with the fusion proteins dRYBP-GST or dRYBPΔNZF-GST. Eluted proteins were analyzed by WB using α-Ub antibody. Note the low amount of ubiquitylated proteins in wild type S2 cell extracts (Input).



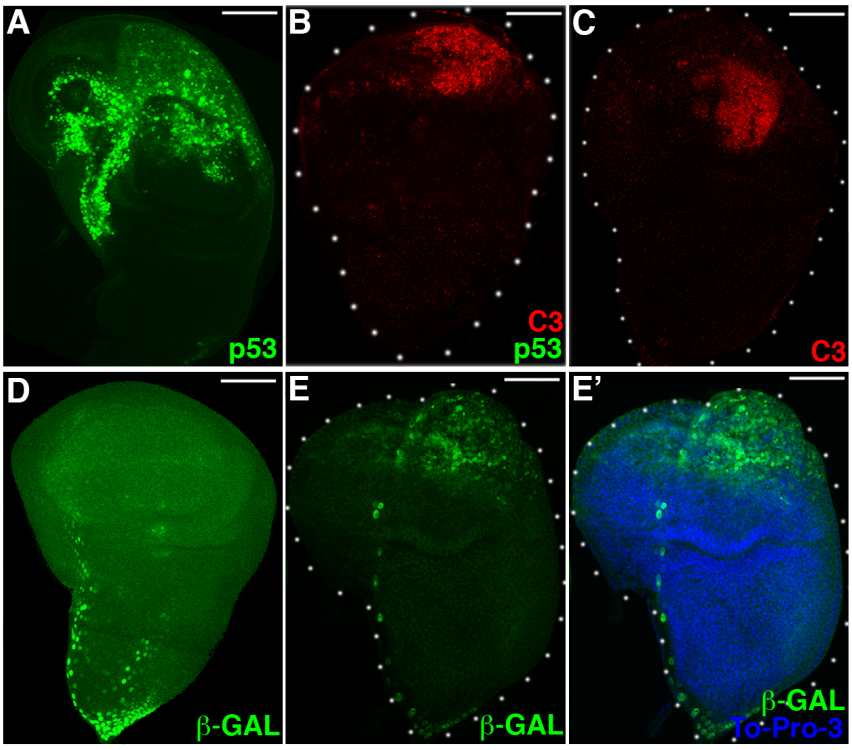
APPENDIX 2. Effect of the inactivation of *Pall* in the wing imaginal disc. (**A**) Wing *sdGal4>UAS-Pall*$_{RNAi}$. Note the wing does not show any visible phenotype. Scale bar represents 0.5mm. (**B**) Wing disc *enGal4>UAS-Pall*$_{RNAi}$/UAS-GFP showing GFP (green) expression, labeling the *engrailed* expression domain in the posterior compartment. (**B'**) Wing disc *enGal4>UAS-Pall*$_{RNAi}$/UAS-GFP showing C3 (red) expression. Note the absence of C3 expression in both wing discs compartments. (**B''**) Merge of images (B) and (B'').



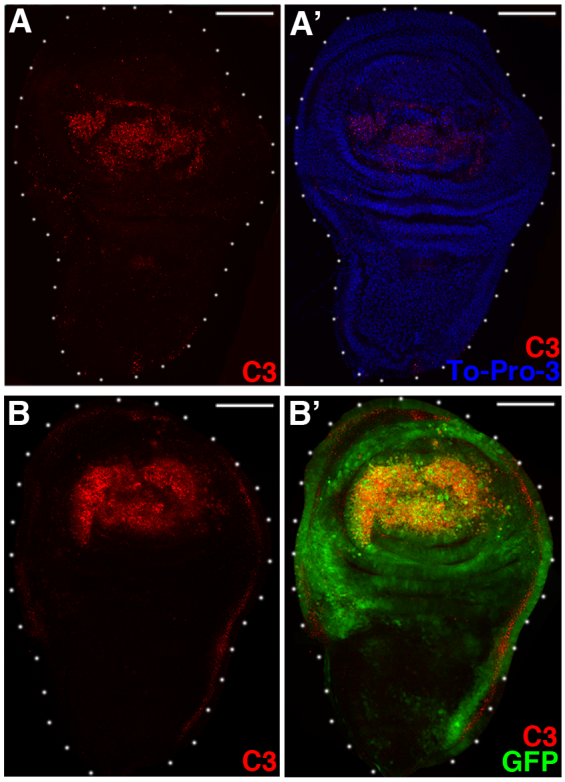
APPENDIX 3. Inactivation of *skpA* does not induce Ubx expression. (**A**) Control wing disc showing wild type *Ubx* (green) expression in the peripodial membrane. (**A'**) Control wing disc in (A) showing To-Pro-3 (blue) expression. (**A''**) Merge of images (A) and (A'). (**B**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$ showing *Ubx* (green) expression. Note cells labeled in green correspond to the peripodial membrane and not the proper disc. (**B'**) Control wing disc in (B) showing To-Pro-3 (blue) expression. (**B''**) Merge of images (B) and (B'). Scale bars represent 100μm.



APPENDIX 4. Overexpression of P35 rescues the apoptosis induced by inactivation of *skpA*. (**A**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing activated-Caspase 3 (C3) (red) and To-Pro-3 (blue) expression. (**A'**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-GFP* showing C3 (red) expression. (**A''**) Enlargement of (A'). (**B**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-p35* showing C3 (red) and To-Pro-3 (blue) expression. (**B'**) Wing disc *sdGal4>UAS-skpA*$_{RNAi}$*/UAS-p35* showing C3 (red) expression. (**B''**) Enlargement of image (B'). Note the differential staining of the a-C3 antibody due to the detection of inactivated C3, inhibited by overexpression of p35 (arrow, detail shown in (B'')) and due to the detection of activated C3, not inhibited by overexpression of p35 (detail shown in (A'')). Scale bars represent 100mm.



APPENDIX 5. Inactivation of *skpA*-mediated apoptosis does not activate p53 expression and induces JNK activation. (**A**) Wing disc *sdGal4>UAS-p53* showing p53 (green) expression, as a control for the antibody staining. (**B**) Wing disc *sdGal4>UAS-skpA$_{RNAi}$/UAS-GFP* showing C3 (red) and p53 (green) expression. Note the absence of cells expressing p53. (**C**) Wing disc *sdGal4>UAS-skpA$_{RNAi}$/UAS-p53$_{RNAi}$* showing C3 (red) expression. Note that the number of apoptotic cells when *skpA* and *p53* are concomitantly inactivated is similar to the number of C3 expressing cells when *skpA* is inactivated (compare (C) and (B)). (**D**) Wing disc *puckered-lacZ* (*puc-lacZ*) showing β-GAL (green) expression. (**E**) Wing disc *sdGal4>puc-lacZ/UAS-skpA$_{RNAi}$* showing β-GAL (green) expression. (**E'**) Wing disc *sdGal4>puc-lacZ/UAS-skpA$_{RNAi}$* showing β-GAL (green) and To-Pro-3 (blue) expression. Scale bars represent 100μm.



APPENDIX 6. High levels of dCUL1 and dRYBP proteins induce apoptosis. (**A**) Wing disc *sdGal4>UAS-dCUL1* showing C3 (red) expression. (**A'**) Wing disc *sdGal4>UAS-dCUL1* showing C3 (red) and To-Pro-3 (blue) expression. (**B**) Wing disc *sdGal4>UAS-dRYBP;UAS-GFP* showing C3 (red) expression. (**B'**) Wing disc *sdGal4>UAS-dRYBP;UAS-GFP* showing C3 (red) and GFP (green) expression. Scale bars represent 100μm.